PROCEEDINGS OF THE SIXTH ANNUAL CONFERENCE

ON

THE NEPHROTIC SYNDROME

EDITED BY

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INTRODUCTORY REMARKS TO THE CONFERENCE

The Sixth Annual Conference on the Nephrotic Syndrome convened at nine-fifteen o'clock in the Millikin Room, Western Reserve University School of Medicine, Cleveland, Ohio, with Dr. Charles Janeway, presiding.

CHAIRMAN CHARLES JANEWAY: I think we had better come to order. For those of you who haven't attended one of these sessions before, the only thing we would like to say is that this meeting owes its success almost entirely, I think, to the fact that it is totally informal and if not disorganized, at least not overorganized.

Dr Heymann has done a wonderful job in getting together a splendid program. He is our host this time. I think he has done a swell job, and we are all very grateful to him for his help

For anybody who hasn't been here before, we are happy to see them here, and particularly the large delegation of hypertension people, who I think are going to teach a lot of us a great deal.

I. FURTHER STUDIES OF THE NORMAL STRUCTURE OF THE RENAL GLOMERULUS

CHAIRMAN JANEWAY. We might as well get right down to business, and we are glad to welcome Dr Hall back for a second go-around on Glomerular Structure. Dr Hall.

DR VINCENT HALL. Thank you, Mr. Chairman I am again honored in being invited back to this group, and I appreciate the opportunity very much to talk to you again about my favorite subject, the glomerulus. I wish to preface this discussion, as last year, by stating what is said today is said as a preliminary and informal introduction to our recent and current studies. Also, as last year, I wish to state that what has been gained in the way of new evidence on glomerular structure has been made possible largely through the support of the Argonne National Laboratory, for the electron microscopy has been done with the capable aid of Evans Roth, electron microscopist, the section cutting has been done by O. T. Minich, technician, and A. S. Tracy, photographer, has been of valuable assistance in obtaining the photomicrographs. Without the able and efficient assistance of these members of the staff of the Biological and Medical Research Division of the Argonne National Laboratory, there would be only a fraction of the evidence now available on glomerular structure. We should also like to use this opportunity to acknowledge the ready and effective cooperation of Dr Hans Popper, Director of the Department of Pathology of the Cook County Hospital, in making human autopsy kidneys and facilities of his laboratory available to us, and to Dr. Johannes Rhodin, Department of Anatomy, Karolinska Institutet, Stockholm, for help and advice in fixing rabbit kidney tissue by his method[1]. Our thanks are also due to Dr. P B Sawin of the Jackson Memorial Laboratory, Hamilton Station, Bar Harbor, Maine, for supplying us with the large, normal, mature male rabbit used as the source of rabbit kidney tissue in this study. All kidneys, the human, the rat, dog and rabbit, used in this study were from mature males, except for the 5-day old rat kidney, and all were judged to be normal, healthy kidneys in every respect.

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[1] Rhodin, Johannes, "Correlation of Ultrastructural Organization and Function in Normal and Experimentally Changed Proximal Convoluted Tubule Cells of the Mouse Kidney," 76 pp., Stockholm, 1954.

Before discussing the illustrations a statement should be made concerning the technics used. Last year [2] many of the tissues that we used for electron microscopy and discussed here were fixed by perfusion, with buffered formalin and formalin-alcohol mixtures, and in all cases our electron micrographs last year had been made after removing the plastic embedding medium from the sections with toluene, as the paraffin is removed from standard histological sections before staining them. For comparison this account includes two electron micrographs made with our earlier methods The remaining electron micrographs have been made from thin tissue slices fixed by immersion in buffered 1% osmium solutions according to the technics described by Palade [3] and Rhodin [1], and the electron micrographs were made without removal of the plastic. Otherwise the methods used in electron microscopy were similar to those described last year.

Some of the current studies to be presented today include direct observation and photography with a Leitz Ultropak microscope of latex injected glomeruli which were partially macerated with acid and then carefully dissected with fine glass needles under the high powers of a dissection microscope. Those preparations consisting of single or a few glomeruli were covered with a cover glass to which slight pressure was applied to obtain a flat field of the capillaries The latex before using was force filtered through a fine 200 mesh per sq. in. stainless steel screen, then diluted and stabilized with ammonia. The latex solution was injected directly into the renal artery or at times the renal vein.

Last year [2] it was emphasized that the differentiated, mature cells of the visceral layer of Bowman's capsule as revealed by the electron microscope do not fit the customary definition of epithelial cells. Since they appear to be uniquely specialized cells found only in the glomerulus, we termed them podocytes (foot-cells), a term descriptive of the numerous, minute characteristic terminal, interdigitating, cytoplasmic process, foot processes or pedicels, developed by these cells. Figure 1 shows the general appearance of a rat podocyte, with its irregular, complex nucleus and a Golgi body of the Hirsch-Baker type near it. The main mass of the podocytic cytoplasm breaks up into many relatively large processes, from a fraction of a micron (10,000 A) to several micra wide and up to 30μ long at least. These large processes were called trabeculae, incompletely pictured here in section on the left of the micrograph and to the right of the podocytic nucleus. It is probably these trabeculae, perhaps emphasized and distorted by vigorous fixation and staining procedures, which the meticulous and able German histologists Zimmermann [4], v. Möllendorff [5], and Bargmann [6] have

[2] Hall, B. Vincent, "Studies of Normal Glomerular Structure by Electron Microscopy," Proceedings of the Fifth Annual Conference on the Nephrotic Syndrome, pp. 1-39, New York, 1953.

[3] Palade, George E., "A Study of Fixation for Electron Microscopy," Jour. Exp. Med., 95: 285-298, 1952.

[4] Zimmermann, K. W., "Uber den Bau des Glomerulus der Saügerniere," Zeitscht. f Mikro. Anat. Forsch., 32: 176-278, 1933.

[5] v. Möllendorff, Wm., "Einige Beobachtungen über den Aufbau des Nierenglomerulus," Zeitscht. f. Zellforsch. u. Mikro. Anat., 6: 441-450, 1927-28.

[6] Bargmann, W., "Weiter Histologische Untersuchungen am Nierenkörperchen," Zeitscht. f. Zellforsch. u. Mikro. Anat., 18: 166-191, 1933.





Fig 1 Electron micrograph showing to the left typical example of the large complex cells podocytes covering the glomerular capillary wall Characteristic are the complex foldings of the nuclear membrane the long and relatively large cytoplasmic processes or [illegible]

osmium X? 000

Fig 2 High magnification electron micrograph of tangential and oblique section through structures forming the "filtration apparatus" Space at upper border with micron marker is intracapillary space (capillary lumen) The numerous large openings are pores of the specialized, thin, cortical endothelial cytoplasm (lamina fenestrata) lining much of the glomerular capillary wall. Through most of the openings of the lamina fenestrata and between some of the dark, finger-like interdigitating pedicels, there appears in very thin tangential section lamina densa (definitive basement membrane) material which in some areas has a characteristic "apparent fine porosity", perhaps merely an artifact, but of dimensions approaching those sufficient to serve as an ultrafilter for proteins Rat glomerulus fixed in alcohol-formalin-osmium X57,000

observed and sketched. Some of our photomicrographs [2] of thin (2μ) sections of rat glomeruli show the trabeculae about 1.5 μ apart extending in a generally parallel fashion away from the region of the podocyte nucleus. Electron micrographs are available which also show the parallel arrangement of trabeculae very clearly. In Figure 1 and also in Figure 4 may be seen pedicels arising from the trabeculae and the proximal surface of the cell body of the podocyte. The terminal relationship of the pedicels on the capillary wall is not clearly shown in either of these figures but may be seen in Figures 11 and 12. However, the external surface of the basement membrane is clearly marked by the presence of numerous pedicels, except in those areas where trabeculae occur. It may be stated here that Pease and Baker [7], and Oberling and co-workers [8] first saw interdigitating ridges in the glomerular capillary basement membrane, but it was Dalton [9] who first recognized in electron micrographs that the "ridges" were minute cytoplasmic processes (pedicels) from the cells of the 'visceral' layer of Bowman's capsule. Before going on, attention should be directed to the two endothelial nuclei on the right, one projecting into the capillary lumen, the other cut near its attachment to the capillary wall so that in this section its projection into the capillary lumen is not seen.

Figure 2 has been included to show an excellent example of a tangential and oblique section of that part of the capillary wall directly concerned with formation of the glomerular filtrate in the rat It is, in fact, a direct plane and oblique view of a small area of the basement membrane of a glomerular capillary such as that which appears in perpendicular view just below the center of the podocytic nucleus in Figure 1. It is apparent in Figure 2 that the structure of the glomerular capillary basement membrane, which appears as a homogeneous dark line when viewed in section with a light microscope, is resolved by the electron microscope into an intimately associated complex of three structures, the pedicels on the capsular surface, the lamina densa or definitive basement membrane forming the continuous dense central layer, and a thin, luminal layer of perforated, cortical endothelial cytoplasm, the lamina fenestrata.

Most of Figure 2 is filled with plane or tangential sections of the interdigitating pedicels, appearing as dark finger-like processes. The space at the top with the micron marker is capillary lumen, with the lining network (lamina fenestrata) with its prominent interstices or pores clearly visible in the capillary lumen and disappearing in the region of the pedicels. Oberling, Gautier and Bernhard's [8] figures and sketch, and the electron micrograph of Rinehart, et al [10] indicate the presence of the thin

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[7] Pease, Daniel C., and Baker, R. F., "Electron Microscopy of the Kidney," Am. Jour of Anat , 87: 349-390, 1950.

[8] Oberling, Charles, Gautier, A., and Bernhard, W., "La Structure des Capillaires Glomerulaires vue au Microscope Electronique," La Presse Medicale, 59[2]: 938-940, 1951

[9] Dalton, A J., "Structural Details of Some of the Epithelial Cell Types in the Mouse as Revealed by the Electron Microscope," Jour. Natl. Cancer Inst., 11: 1163-1185, 1951

[10] Rinehart, James F., Farquhar, M. F., Jung, H. C., and Abul-Haj, S. K., "The Normal Glomerulus and Its Basic Reactions in Disease," Am. Jour. of Path., 29: 21-32, 1953.

fenestrated endothelial membrane in rat glomerular capillaries. The French workers interpreted it as a "honeycomb reinforcement" to the endothelial membrane, and the San Francisco workers pictured this fenestrated membrane as if it were formed by delicate cytoplasmic projections of epithelial cells penetrating the basement membrane, a mucoid substance which traverses the basement membrane "at regular intervals in a manner analogous to the cement lines separating hexagonal tile." The gray material appearing in some of the openings of the lamina fenestrata and appearing between some of the pedicels (as near the bottom of the picture and left of center) is a thin section of the definitive basement membrane or lamina densa. This complex basement membrane with its three separate layers, it seems to us, must function as the filtration apparatus in glomerular filtration. Since the pores of the lamina fenestrata are too large (in this section their diameters measure from about 700 to 1500 A) to retain proteins, so it must be assumed either that the pores are artifacts, or that they are not artifacts and that they function otherwise than in the retention of proteins in filtration. Last year [2] we suggested that numerous and large pores in the lamina fenestrata (specialized, thin, perforated glomerular endothelium) promote the high filtration rates characteristic of glomerular capillaries by removing the endothelial cytoplasmic barrier and providing ready access of plasma to the definitive basement membrane (lamina densa). The lamina densa we suggested served as the ultrafilter since it appeared to be the only structure in the capillary wall sufficiently continuous and possibly finely porous to retain plasma proteins. The frequent and regular appearance of a fenestrated lining endothelium in our electron micrographs of rat, rabbit, and human glomeruli, and the duplication of these observations by Pease [11] in rat glomeruli, by Mueller [12] in dog glomeruli, and Dalton [13] and Rhodin [14] in mouse glomeruli affords support to the suggestion that pores in the lamina fenestrata function in glomerular filtration. However, it is to be noted that the concept of functional endothelial pores will have to be abandoned, if future studies confirm the recent suggestion of Rinehart and Farquhar [15] that fluid transfer across the endothelial cytoplasm of the glomerular capillaries takes place "via vesicles as suggested by Palade [16] for certain other endothelia." Since the evidence seems to be good [17] that glomerular filtrates are normally

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[11] Pease, Daniel C., "Further Studies of the Kidney Cortex by Electron Microscopy," Anat. Rec., 118: 339-340, 1954.

[12] Mueller, C. B., "Oral Communication, Symposium on Protoplasmic Structure and Cellular Transport Mechanisms," The Mount Desert Island Biological Laboratory, Salisbury Cove, Maine, August 23, 24, 25, 1954.

[13] Dalton, A. J., "Oral Communication, Symposium on a New Look at the Cell," Annual Meetings of the American Society of Zoologists, Chapel Hill, North Carolina, December 28, 29, 30, 1954.

[14] Rhodin, Johannes, "Oral Communication, Symposium on Protoplasmic Structure and Cellular Transport Mechanisms," The Mount Desert Island Biological Laboratory, Salisbury Cove, Maine, August 23, 24, 25, 1954.

[15] Rinehart, James, F., and Farquhar, M. G., "Functional Implications of the Fine Structure of the Renal Glomerulus," Applied Physics, 25: 1463, 1954.

[16] Palade, George E., "Fine Structure of Blood Capillaries," Applied Physics, 24: 1424, 1953.

[17] Smith, Homer W., "The Kidney: Structure and Function in Health and Disease," 1049 pp., Oxford University Press, New York, 1951.

relatively protein-free, it appears that such a cytoplasmic vesicular transport mechanism must also provide a means to return unfiltered proteins back to the plasma, unless the vesicles somehow transport only protein-free plasma to the definitive basement membrane. Certainly for the present it seems that the weight of evidence from electron microscopy of glomerular capillaries, and the great mass of renal filtration data [17, 18] favor a "pore theory" of renal filtration. In fact, the physiological evidence such as that which led Richards [19] to state, "that the beginning of urine formation consists in the separation from blood of a torrent of undifferentiated filtrate by a blind physical force" would appear to make it improbable that an active cytoplasmic transport process is involved.

Near the center and bottom of Figure 2 may be seen between two pedicels "an apparent fine porosity" in the thin tangential section of lamina densa. As we emphasized last year [2], these apparent holes may be artifacts pure and simple, yet they do have apparent diameters which approach those which theoretically permit the ready passage of egg albumen molecules but only restricted passage of hemoglobin molecules, conforming with the known facts of glomerular permeability. Evidences of such "an apparent fine porosity" have not been seen in perpendicular sections of lamina densa, nor has there been any clear evidence for such fine porosity in our electron micrographs of sections fixed in buffered osmium and photographed without removal of the plastic embedding medium. So at present, until more evidence is available, we feel that two opinions may be held, one that the apparent fine porosity is solely an artifact, the other that some technics, which result in pictures showing a fine porosity as in Figure 2, have exaggerated the appearance of a possibly naturally existing fine porous structure of the lamina densa, while other technics, such as those used in obtaining Figures 5 and 12 decrease, and perhaps occlude, the fine porous structure of the lamina densa.

The sketch shown in Figure 3 has been drawn to scale from electron micrographs of rat glomerular capillaries and gives some idea of the relatively large size of a podocyte (which is only incompletely shown here) and endothelial cells. The ratio of 3.1 is about the true ratio of endothelial cells to podocytes, for the rat at least, and the inverse ratio approximately corresponds to their comparative sizes. One important feature to be noted in this diagram is that each endothelial nucleus is embedded in dense medullary cytoplasm and that the medullary cytoplasm around one nucleus joins closely with that around the next nucleus, but as was shown in Figure 8 last year [2], the cytoplasmic membranes remain distinct and the cells separate so the arrangement probably should not be called a syncytium. It is clear from study of our series of photomicrographs of 2 μ serial sections of rat glomeruli and extensive photomontages of detailed electron micrographs, that the glomerular endothelial nuclei with their surrounding medullary cytoplasm are polarized in a band or row along one side of each capillary, with the centers of the nuclei about 6-10 μ apart. We remain of the same opinion expressed last year [2] that these bands of nuclei and individual endothelial nuclei, with the surrounding dense medullary cytoplasm, have at times been misinterpreted for a special type of glomerular connective or supporting tissue, the mesangium

[18] Pappenheimer, John R., "Passage of Molecules Through Capillary Walls," Physiol. Reviews, 33: 387-423, 1953.

[19] Richards, A N., "Processes of Urine Formation," Proc. Roy. Soc. Ser. B., 126 398-432, London, 1938-39.

or interstitial cells. Bell [20] and other pathologists have stressed the importance of changes in the glomerular endothelium in renal disease, if, as the evidence of the electron microscopy leads us to believe, the glomerular capillaries are formed and supported by only two types of cells, podocytes and endothelial cells, and their derivatives, then the proliferation and changes in the endothelium in disease have probably at times been described as changes and proliferation of the mesangium or interstitial cells. We again find no evidence for an "intercapillary space" in the special sense of McManus [21], for all glomerular space appears to be either intracapillary (capillary lumen) or extracapillary (capsular or Bowman's space) under the electron microscope. In fact, as it will be demonstrated in subsequent figures today, new evidence of the total morphology of the glomerulus and facts of its development make it highly probable that simple capillary loops, as diagramed by Vimtrup [22], occur in the mammalian glomerulus only rarely if at all, and as a consequence it appears that the idea that such simple capillary loops constitute the organizational basis of glomerulus will have to be abandoned. If this proves to be true as the evidence strongly indicates, then the conceptual basis used by Zimmermann [23] and others to develop the idea of a glomerular mesangium, interstitial tissue, and "intercapillary space" is erroneous, leaving the mesangial concept as it applies to normal glomeruli of the rat, dog, rabbit, and man, unsupported in fact and theory. It would appear that the whole concept of "mesangium", etc., has developed as a result of the inadequacy of the light microscope to clearly resolve the complexities of glomeruli viewed in sections.

Figures 4, 5 and 6 are relatively high magnification electron micrographs of the surface and internal structure of the basement membrane of the rat glomerular capillary Figure 4 has been selected to illustrate interdigitating pedicels, attached to the large trabecular processes of the covering or podocyte cells. Of particular interest in *this figure is the clearly apparent, rather uniformly narrow (about 200-400 A) space* between the pedicels which represents the smallest direct extension of capsular space to the surface of the definitive basement membrane, the lamina densa. It is probably here in this space that the glomerular ultrafiltrate first enters free capsular space. The dark bodies in the trabeculae are mitochondria, but we have yet to observe mitochondria in the pedicels.

In Figure 5 may be seen in oblique and tangential section, and in Figure 6 below the micron marker in perpendicular section the lamina fenestrata with its characteristic pores or holes. We hope to obtain clearer electron micrographs of this region in the rat, well fixed with buffered osmium and photographed without removing the plastic from the section, but we have none better now. Comparison of these figures with Figure 2 shows the same structures, lamina densa, pedicels, and lamina fenestrata appear in the figures, but it is to be noted that the pores of the lamina fenestrata appear

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[20] Bell, E. T., "Renal Diseases," 448 pp., Lea and Febiger, Philadelphia, 1950.

[21] McManus, J. F. A., "Medical Diseases of the Kidney," 176 pp., Lea and Febiger, Philadelphia, 1950.

[22] Vimtrup, Bj., "Number, Shape, Structure, and Surface Area of Glomeruli in Man and Animals," Am. Anat., 41: 123-151, 1929.

[23] Zimmermann, K. W., "Uber den Bau des Glomerulus der menschlichen Niere," Zeitscht. f. Mikr. Anat. Forsch., 18: 520-552, 1929.

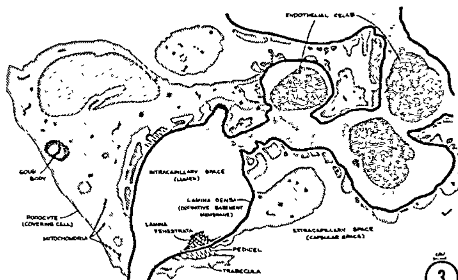




Fig. 3. Sketch of a section through glomerular capillary showing relative size of podocyte (covering cell) and endothelial cells. Large podocytic processes or trabeculae and their discrete minute interdigitating terminal processes or pedicels attach intimately to the external surface of the lamina densa (definitive basement membrane). The endothelial nuclei are embedded in a band of dense, medullary cytoplasm polarized along one side of the capillary while elsewhere the capillary is lined by a thin layer of perforated, endothelial cortical cytoplasm, the lamina fenestrata.



Fig 5 Electron micrograph at high magnification of lamina [illegible] apparent size of the interstices (pores) of the lamina fenestrata are smaller With this technic it has not been possible to visualize an "apparent fine porosity" in the lamina densa with any certainty Rat kidney fixed in buffered osmium X62,000

smaller and the lamina densa appears structureless in Figures 5 and 6. In comparing dimensions of pores, and of the cytoplasmic strands between the interstices of the lamina fenestrata in the two types of electron micrographs, it is apparent that cytoplasmic shrinkage directly affects the apparent size of the pores. In Figures 5 and 6 both the shrinkage and the apparent pore size are less than in Figure 2. The technic of fixation, i.e., whether the fixative was given by perfusion or immersion, and the type of fixing fluid both seem to influence the apparent thickness of the lamina densa. In some preparations fixed with formalin-alcohol by perfusion, the lamina densa is as thin as 500 A, while in Figure 6, it appears about 4 times as thick. These facts emphasize and warn us of the danger and the difficulty that there is in any attempt to relate what we see in electron micrographs to the entity functioning in life. In spite of the possibility that fixation has destroyed an ultrathin membrane which covers the pores of the lamina fenestrata in life, the evidence of perpendicular sections such as in Figure 6, makes it difficult to draw any conclusion except that it is possible that open pores in the lamina fenestrata are present in life.

With Figures 7 and 8, we begin the discussion of [illegible]
glomeruli These tissues were fixed with buffered
directions and photographed with the plastic still in the
is a typical example of a rabbit podocyte. Two trabeculae sectioned in their long axis are present on the left and several obliquely sectioned trabeculae appear below the podocyte near the basement membrane. The capillary lumen is the space at the lower edge of the figure Close inspection of the basement membrane reveals its three characteristic layers, the inner luminal layer of thin, perforated endothelial cytoplasm, the lamina fenestrata, in the middle the continuous dense definitive basement membrane or lamina densa, and externally the numerous, minute, terminal podocytic processes the pedicels or foot processes. In general the podocytes of the rabbit glomeruli (excepting perhaps the 'Golgi body') are much like those of rat and human glomeruli.

It was suggested last year at this Conference in Philadelphia [24], that our failure to find intercapillary space was probably due to the fact that we had worked with rats, and it was implied that a similar study of rabbit glomeruli would reveal evidence of a connective tissue supporting the capillary loops. Figures 7, 8, 10, 11 and 12 are submitted as typical examples of the structure of the rabbit glomerulus as viewed with the electron microscope. After intensive study of these electron micrographs, and many similar ones, we have not been able to identify connective tissue or collagen fibers in the normal rabbit glomerulus. In fact, we find that rabbit glomeruli are basically similar to rat glomeruli in possessing (ignoring blood cells) only two types of cells, podocytes and endothelial cells, with their specialized processes and derivatives. Just as close examination of Figure 7 gives no evidence of connective tissue, but only lamina densa with the podocyte and podocytic processes on the capsular surface, and endothelial cytoplasm on the inner surface so does similar close examination reveal no connective tissue or connective tissue cells in Figure 8. Three endothelial cells appear in Figure 8, sectioned near their attachment to the capillary wall.

[24] Ehrich, Wm and Piel, Carolyn, "Morphologic Differentiation of Nephritis in the Rat and the Therapeutic Effects of Anticoagulants and Proteolytic Enzymes," Proceedings of the Fifth Ann. Conf. on the Nephrotic Syndrome, pp. 117-134, New York, 1953.

Each nucleus is surrounded by an envelope of dense medullary cytoplasm, while away from the region of the nucleus (shown more clearly in Figure 11) the medullary cytoplasm gives way to a thin layer of perforated cortical cytoplasm or lamina fenestrata. It may be seen in Figure 8 lining the capillary lumen just above the middle one of the three endothelial nuclei. External both to the lamina fenestrata and to medullary endothelial cytoplasm there is present always lamina densa, or definitive basement membrane, which here appears as a continuous light and dark gray membrane. This electron micrograph is apparently through a region of the capillary which has been folded (near the bottom of the picture and to the left of center) and where branching or anastomosis occurs, for the lamina densa can be traced in a rather complicated but fully understandable pattern. On the external surface of the lamina densa is found only podocytic cytoplasm, specialized either into pedicels or trabeculae. It should be noted before leaving this illustration that the endothelial nuclei in this figure seem to illustrate clearly the typical arrangement described for rat glomerular endothelium, with the nuclei located 6-10 μ apart, in a band of medullary cytoplasm polarized along one side of the capillary.

In the schema of Figure 9 is shown the relationship of the three layers in the glomerular capillary wall, which must together comprise the "filtration apparatus". To help in recognizing the size relationships a portion of an endothelial nucleus has been included. It will be noted that the cytoplasmic envelope around the nucleus thins to give rise to the specialized, perforated, cortical cytoplasmic layer, the lamina fenestrata away from the region of the nucleus. The endothelial cytoplasm is in intimate contact with the possibly finely porous lamina densa. Its intimate relationship to endothelial cytoplasm, and the close relationship of endothelial nuclei to this membrane, and certain facts of its development lend support to the opinion that the origin of the lamina densa is endothelial. On the external surface of the lamina densa may be seen in surface view on the left and in perpendicular section on the right, the pedicels, characteristic terminal, interdigitating processes of the podocytes. At this scale a single large podocyte would extend completely over a surface having perhaps 30 times the area of the schema, so a small part of only two larger podocytic processes can be shown here. If the actual filtration area is limited to the free surface of the lamina densa between the pedicels, which communicates directly and freely with capsular space then it seems quite possible that pathological, physiological, and local environmental effects may induce the pedicels to extend or retract over the surface of the lamina densa and cause related changes in glomerular filtration rates.

In Figures 10, 11, and 12 are illustrated relatively high magnification electron micrographs of details of the interdigitating pedicels (Fig. 10) of the rabbit as viewed in a plane or tangential section, corresponding to the left side of the schema in Figure 9. They appear in every way to be similar to those of the rat (Fig. 4). In the lower right corner of the figure may be seen a mitochondrion with its cristae barely visible. Mitochondria are regularly observed in the larger podocytic processes such as these, but they have not been observed in the pedicels.

The close relationship of the endothelial nucleus in Figure 11 to the lamina densa (definitive basement membrane) is very evident. This figure also shows pores of the lamina fenestrata in tangential section at the top right and lower left, and in perpendicular section to the left of the nucleus and at the lower right. The lamina densa is





Fig 7 Electron micrograph of typical rabbit podocyte (covering cell) on surface of glomerular capillary with characteristic irregular nucleus mitochondria and two prominent elongated proc- [illegible]

Fig 8 Electron micrograph of 3 typical rabbit endothelial cells sectioned near their attachment to the glomerular capillary wall The nuclei often appear in near contact with the lamina densa (definitive basement membrane) though usually a thin layer of dense medullary cytoplasm with small mitochondria may be seen enveloping the nucleus Away from the nucleus the cytoplasm thins, to the specialized layer of cortical perforated cytoplasm (lamina fenestrata) seen lining the capillary lumen left of center near top of picture At bottom of picture to left of center the section passes in and out of the lamina densa of a folded capillary wall. At adequate magnifications of this and similar electron micrographs, it appears that within the normal glomerulus, excepting blood cells, there can be found only endothelial cells and podocytes (covering cells) and their processes and products, trabeculae, pedicels, medullary cytoplasm, lamina fenestrata and lamina densa Fixed in buffered osmium X7 700

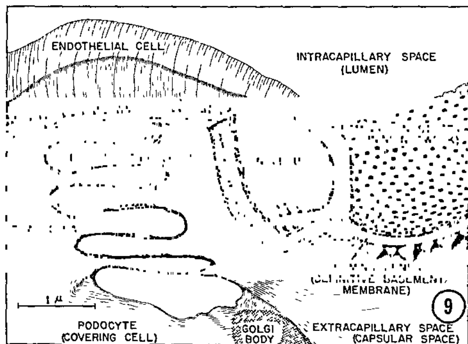




Fig 9 Schema showing relationship of structures forming the "filtration apparatus" of the glomerular capillary wall In regions away from the nucleus endothelial cytoplasm thins to a perforated layer of cortical cytoplasm whose relatively large pores permit [illegible]

proteins and escapes directly into minute extensions of capsular space found between the interdigitating pedicels Dimensions and frequency of pores drawn in the lamina fenestrata are both provisional and approximate and are maximal values based on technics which appear to exaggerate the apparent porosity

Fig 10 Electron micrograph showing interdigitation of pedicels as viewed in tangential section of glomerular capillary wall This group of pedicels appears to have its origin from 4 *secondary podocytic processes* or trabeculae which appear in upper left, lower right and top and bottom of the picture Rabbit kidney fixed in buffered osmium X39 000

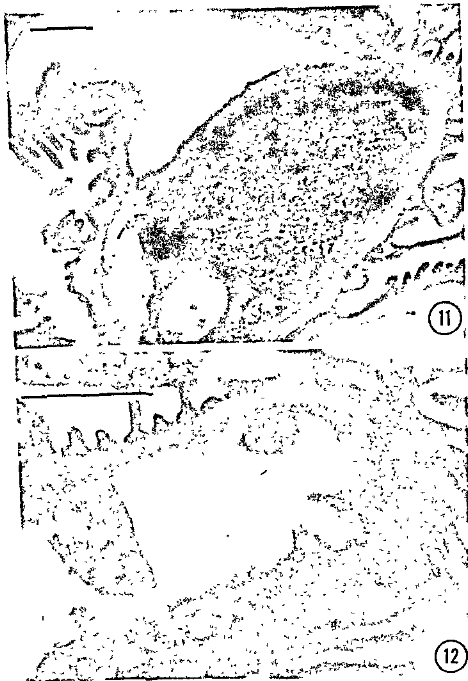


Fig. 12. Electron micrograph of perpendicular section (above) and oblique and tangential section (below and right) of pedicels, *lamina densa* and *lamina fenestrata* which together function as the "filtration apparatus" and which cannot be separately visualized by the light microscope. The glomerular basement membrane viewed with a light microscope must include to some variable degree all 3 structures so clearly delimited in adequate electron micrographs of perpendicular sections of the basement membrane of glomerular capillaries. Diameters of pores in the lamina fenestrata vary from about 400 to 1000 A in this section, and their *maximum frequency* seems to be about 5×10^9 per cm^2. Central space intracapillary space, all other space extracapillary (capsular) space. Rabbit glomerulus fixed with buffered osmium ×38 000.

completely covered externally by pedicels, some sectioned perpendicularly, some obliquely, and some longitudinally. In Figure 11 and in Figure 12 below it, it is to be noted that there is not the slightest indication of any connective or mesangial tissue associated with the rabbit glomerular capillary wall.

A good illustration of lamina fenestrata with its pores, in tangential section, and of pedicels obliquely and perpendicularly sectioned is found in Figure 12. It appears that the perpendicular profile of a pedicel in the rabbit, as in the rat, may be roughly triangular or oval with a broad flat base attached to the lamina densa. As in the rat, their height appears to be about 1500 A. In this section the pores of the lamina fenestrata vary from about 400 to 1000 A in diameter, and they have a maximum frequency of about 5×10^9 per cm^2. The remarkable similarity in the apparent frequency of the endothelial pores observed in rat and rabbit glomeruli, and (neglecting shrinkage effects for the moment) the equally remarkable similarity in the approximate size of these pores after various fixatives and technics affords the strongest support for the viewpoint that they are not mere fixation artifacts.

With this remark we end the discussion of the electron micrographs

CHAIRMAN JANEWAY Does anybody have any questions?

DR. DAVID GITLIN Do you think the podocytes or their pedicels might conceivably be connected between two capillary areas. I don't say loops necessarily, but between two areas of a capillary

DR HALL: Do you mean between two separate capillaries?

DR GITLIN No If you have a capillary which is tortuous and doubled back on itself, will the pedicels and podocytes conntect between those two?

DR HALL At present I don't think so, but the question cannot be finally answered without serial sections and I may have to recant on this. Since last year, we were lucky enough to get one series of 20 serial sections of which 12 were useful ones. Every idea presented here has been supported by these sections, but they do not give a final answer to your question Embryology says no to your question, because the cells are small to begin with, and as the capillary enlarges the podocyte enlarges What opportunity is there going to be for the podocyte to let loose here and nab there? It is like having a little picture on a balloon that's deflated. Blow up the balloon and the picture gets big, but it always stays in the same relative area. I don't think a podocyte leaves one area or capillary and attaches to another

DR GITLIN: I don't mean that it migrates. I mean, in situ, does one podocyte attach between two capillaries or two areas of one recurrent capillary?

DR HALL I don't think so, but I really don't know. Often it can be seen that the upper surface or capsular surface of the podocyte has no pedicles on it.

DR GITLIN: Then you would say every capillary would have a capsular surface and every wall of the capillary would be free in capsular space?

DR. HALL: Yes. Every podocyte may have a proximal surface with pedicels in contact with the lamina densa and a smooth capsular surface like the back of my hand which is exposed to capsular space.

DR GITLIN: Every surface of the wall of the capillary then would also have a capsular surface.

DR HALL For 360 degrees the glomerular capillaries appear to present to capsular space a surface covered by pedicels and trabeculae of the podocytes.

DR SIMON KOLETSKY: I think it hardly need be said that these studies or photographs are a lot different than the average pathologist sees in his everyday work. When one looks at normal fixed tissue he sees, I suppose, a crushed glomerulus with capillary loops with the epithelium closely applied and so forth. However, as you know, we are always worrying about whether a disease process is in the capillary, between capillary loops, or around the capillary. In other words, we are talking about intercapillary, intracapillary, pericapillary, disease.

While it may be true that in a normal glomerulus there is close application, apparently of the epithelium to the endothelium, I think it certainly is true that in disease processes, there appears to be a spread and a true intercapillary space.

In fact, we often think that there are two basement membranes, one for the capillary and one for the epithelium, and that many disease processes are the result of exudative processes which go out of the capillary and localize in this nebulous intercapillary space.

We see a lot of proliferative change in this potential capillary space, and this material surely looks fibroblastic. It doesn't look endothelial, and I am just curious to know whether any studies that you have made bear on pathogenic change in the sense that there are two basement membranes. There is, at least, the potential intercapillary space. There is possibly a true intercapillary space. There is a definite mesangium which I think the great majority of pathologists accept.

DR. HALL: Being an ordinary anatomist and physiologist I am on dangerous ground in attempting to reply to a question relating to pathological glomeruli, with which I have no experience. I can only say that a completely honest and open-minded study of our extensive series of electron micrographs and 2 μ serial sections of rat, rabbit and human normal glomeruli affords absolutely no evidence for the highly controversial glomerular connective tissue termed mesangium, or its nebulous counterpart intercapillary space. On the other hand, the evidence which is offered to support the mesangial theory, at least as far as normal glomerular structure is concerned, appears to us as completely unconvincing and inadequate. All I have to say in this respect is, look at the evidence of the electron micrographs such as you have seen here, and then look at the dark, often black, ill-defined masses of cells and cellular materials, hardly recognizable as such, which have been used to prove the presence of a mesangium, and decide which seems to be the best evidence. Another point which should be made, is that Zimmermann's [23] ideas of a mesangium were based to a considerable extent on his belief in the reality of simple glomerular capillary loops

as described by Vimtrup [22]. We now have good evidence which no one as yet has questioned that at least as far as the rat is concerned, and it also seems true for the dog and man, that there are no simple capillary loops. If this is substantiated, then the mesangial concept is left without a conceptual basis.

To answer your question specifically, Dr Koletsky, we have not one electron micrograph in all of nearly 1000, which in any way could be considered as evidence for the presence of two basement membranes or connective tissue within the glomerulus. But at the same time, it should be pointed out that all our material has been of normal glomeruli. Perhaps, pathological glomeruli do have two basement membranes. All we can say is, that we are completely unable from knowledge of normal glomeruli to understand how or where a second basement membrane may develop in diseased glomerular capillaries.

DR NORMAN KRETCHMER I just wanted to ask what method of fixation you used in these pictures

DR HALL *Actually, two methods of fixation were used. The tissues represented* by Figures 1 and 2 were fixed with formalin-alcohol buffered at pH 7 4, and the remaining tissues in 1% buffered osmium solutions.

DR KRETCHMER: You don't feel the formalin fixed tissues produced any artifacts?

DR HALL Well-fixed osmium treated tissues give what is accepted to be the best pictures, but it is probable that all fixatives induce artifacts o some sort. Formalin may produce more and different artifacts than osmium, but formalin and fixatives other than osmium, when properly used, may give useful preparations in electron microscopy It must be admitted that fixatives do give artifacts, and formalin is generally considered less satisfactory than osmium in this respect. Our big problem is to know just what artifacts are meaningful, and what is the meaning of the artifacts.

DR JACK METCOFF I have had the opportunity to see some of your recent studies, and I wish you would escort all of us through the capillary circulation within the glomerulus with your dissecting needle and camera.

DR. HALL There are men here who have more interesting and more important things to present than I have.

CHAIRMAN JANEWAY. Don't apologize. Go right ahead.

DR HALL. The remainder of our discussion today deals with studies undertaken to gain a clearer understanding of the nature of the total morphology of the glomerular capillaries and the glomerulus Since Vimtrup's [22] studies about 30 years ago, it has been generally accepted in the textbooks that the glomerulus is organized as a tuft of simple capillary loops branching directly from the afferent arteriole and without anastomoses terminating separately in the efferent arteriole. We recognized in our electron micrographs last year [2] that the glomerular capillaries made frequent anastomoses, but we were not sufficiently impressed to make a thorough study of this

until we had obtained and studied overlapping electron micrographs of good glomerular sections, which were fitted together as photomontages. Repeated study of these photomontages as well as thorough study of an extensive series of enlarged photomicrographs of 2 μ serial sections forced us to investigate the total morphology of the glomerular capillaries with new methods (new to us, but old in the history of the glomerulus). We decided to inject kidneys with latex as More and Duff [25] and others had done, then after partial acid maceration to dissect well-injected glomeruli with fine glass needles and to study them with the highest powers of the dissecting microscope. We soon learned to put cover glasses on the better preparations and to photograph them with a Leitz Ultropak microscope.

The first figure in this series, Figure 13, is a relatively low power view of several glomeruli with Bowman's capsules removed, as they appear on the surface of a rat kidney It is clear that the afferent arterioles branch into a few basal branches, and these in turn branch again, much as Bowman drew them 112 years ago [26]. Indeed, in several glomeruli, as in the one on the right, it appears that the second order branches (direct communicating vessels) give off small lateral capillaries which apparently anastomose with each other and the second order vessels. Of course a major difficulty in this type of preparation is that the curvature of the ball-like glomerulus is so great that it is next to impossible to obtain satisfactory photographs at sufficient magnifications to be useful Another objection is that only the surface course of a few of the capillaries is visible, even in the best of these preparations. So, we removed an individual latex injected glomerulus from the kidney, dissected off Bowman's capsule, separated, unfolded, and arranged the lobules for maximum visibility, and then flattened the preparation with a cover glass for photography as shown in Figure 14. In these dissected preparations it is often possible to follow the full course of the blood pathway from the afferent to efferent arterioles. In Figure 14, the afferent arteriole is in view end-on just slightly to the left and below the center of the picture where the two lower lobules join. It is typical for the afferent to divide as shown in this figure, into several very short basal branches (2 or 4 here, and 3 in Fig. 15). One basal branch apparently supplies one lobule only. The basal branches divide immediately into a few (usually 3 or 4) direct, communicating vessels which seem to traverse the full length of a lobule. The communicating branches appear to afford direct, and probably preferential pathways for the flow of blood from afferent to efferent arterioles. At the distal end of the lobule where these direct, communicating vessels generally appear reduced in diameter, the lobule tapers to terminate in another very short vessel which joins with the similar vessels from the other lobules of a glomerulus to form the typically small-diameter efferent arteriole. In Figure 16 a direct communicating vessel can be traced from the afferent arteriole directly upward to the top of the small center lobule in the dissection, where it turns to the right, reduces in diameter and joins small vessels which together appear to give rise at this point to the small efferent arteriole which extends further to the right The efferent arterioles are so small and 'undistinguished' that to be completely certain that a particular small vessel in a dissected glomerulus is the

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[25] More, R. H., and Duff, G. L., "The Renal Arterial Vasculature in Man," Am. Path., 27. 95-117, 1951.

[26] Bowman, Wm., "On the Structure and Use of the Malpighian Bodies of the Kidney, with Observations on Circulation through that Gland," Phil. Trans. of the Roy. Soc. of London, 132: 57-80, 1842.

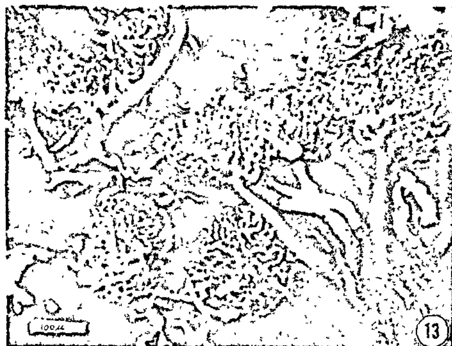




Fig 13 Photomicrograph of latex injected glomeruli on surface of cat kidney after acid maceration partial dissection and removal of Bowman's capsules The afferent arterioles on the right can be seen giving rise within glomeruli to a few short basal branches each of which supplies one lobule making the lobule an anatomical and functional unit of the glomerulus Each basal branch subdivides into a few smaller but relatively large main communicating vessels

Fig 14 Photomicrograph of dissection of latex injected rat glomerulus viewed with lobules flattened and extended



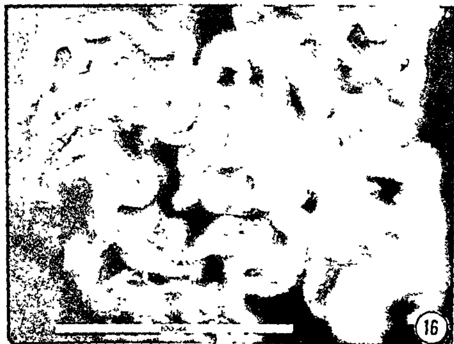


Fig. 15. Photomicrograph of dissection of latex injected rat glomerulus showing the branching of the afferent arteriole into three [illegible]

partially injected but it is fully flattened and shows anastomoses of several branches arising from the main communicating lobular vessels. X305

Fig. 16. Photomicrograph of part of a dissected lobule from a latex injected rat glomerulus. From the lower right and extending for over 200 μ across the top of the picture is an example of one of the main communicating lobular vessels [illegible]

efferent arteriole is difficult. Positive recognition of the efferent arteriole in a dissected glomerulus is made possible only by recognition of the efferent arteriole before beginning the dissection, which is usually easily done. Then, it must be retained in sight while the actual dissection is made, which sometimes is difficult to do, and finally completely accurate notes of the dissection must be kept to positively identify the vessel in the photograph later.

After close examination of Figure 14 and especially after study of Figures 16 and 17, it cannot be doubted that the direct communicating vessels within the lobules branch freely, seemingly at right angles to their axis, to give rise to numerous usually smaller diameter and often short capillaries. These lateral branches anastomose with each other and with the direct communicating vessels to form a true capillary network within the confines of each lobule of the glomerulus. The total evidence of our studies seems to establish that at least for the rat, the lobule is the anatomical and presumably the functional unit of the glomerulus. We have extended these studies to glomeruli of the dog, Figure 18, and man, Figure 19, as well as to the rabbit and cat Although species differences are present, the total capillary organization of glomeruli in these forms appears, in general, basically similar.

Certainly the photomicrographs presented here of dissected, latex-injected glomeruli clearly establishes that glomerular capillaries in the rat, dog, and man anastomose freely, and that glomeruli in these forms are not composed of simple capillary loops connecting directly between the afferent and efferent arterioles. Other dissections of dog and human glomeruli are equally clear and demonstrate a system, similar to that described and pictured for the rat, of direct communicating glomerular vessels within the lobules, leading directly to the efferent end of the lobule and giving rise laterally to numerous smaller diameter anastomosing capillaries.

Before commenting briefly on preliminary studies of the embryology of glomeruli made with the electron microscope, I'd like to point out that we are not the first to have seen and to be impressed with the lobular capillary network organization of mammalian glomeruli Over 80 years ago, Carl Ludwig [27] saw, described and sketched the total organization of glomerular capillaries in a way which differs in no basic point from the detailed photographic evidence presented here.

Also it may be well to use a little time for discussion of the functional implications of these studies. It is possible that the organization of the glomerular vessels into relatively direct communicating channels in an associated capillary network of smaller anastomosing vessels may in some way facilitate filtration. Perhaps it affords a structural basis for the skimming of plasma relatively freed of cells into the network of small capillaries, while the great mass of blood cells directly and rapidly flows through the lobule to the efferent arteriole as an axial stream. In the network of smaller capillaries the rate of flow may be less, and as a result turbulence reduced.

It may also be mentioned that these studies make a comparison of the capillaries

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[27] Ludwig, Carl, "The Kidney," Chap. XXI, pp 460-490, A Manual of Histology by Prof. S Stricker, Wm Wood and Co., New York, 1872.

of a glomerulus with those of a tissue, like the mesentery, more possible than has hitherto been realized. In fact, the similarity between the preferential or thoroughfare capillaries, described and pictured in rat mesenteries by Chambers and Zweifach [28] giving rise at right angles to small diameter capillaries, to some of the direct communicating vessels that we have photographed in dissected glomeruli is striking.

It is of interest to note that Trabucco and Marquez [29] have recently also concluded from a study of injected glomeruli of the rabbit, dog, and man, and from analysis of the physiology of renal circulation, that the glomerular tuft is not composed of "loop-formations, as described by Vimtrup." At the same time we hasten to emphasize that our dissections do not support their statements that "the glomerular bundle ends in saccular (blind) tubular arrangements", without anastomoses, and that the afferent arteriole after entering the glomerulus continues immediately and directly as the efferent arteriole. A careful examination of their published photographs leads to the view that their impressions resulted from the study of incompgetely injected or visualized glomeruli, and from a misunderstanding of true relationship of the vessels demonstrated. Most of their photographs demonstrate only the relatively large, direct communicating vessels arising from the basal branches of the arteriole. In their photographs these vessels APPEAR (as they do in some of our figures) to end blindly for the simple reason that they were incompletely injected and their small diameter terminal endings joining the equally incompletely injected efferent arteriole are not demonstrated. Our universal experience in making many detailed dissections of well-injected glomeruli of the rat, dog, and man as we have demonstrated here today, has been that the afferent vessel subdivides into several direct communicating vessels, which give rise to numerous anastomosing small capillaries within the lobules. Each lobule folds over back on itself near the tip of the glomerulus, opposed to the vascular pole, and with the capillaries reduced both in size (at times) and number, the lobule tapers back to the vascular pole, where with a single connection (in the rat at least) it joins an inconspicuous efferent vessel. It is impossible to entertain a reasonable doubt that blood circulates from the afferent, through the glomerular capillaries, and into the efferent arteriole.

I am sorry to be using so much of your time, but there may be questions.

CHAIRMAN JANEWAY: Does anybody have any comments or questions they would like to ask? I think, Dr. Hall, you have convinced us so that nobody either knows enough or can think fast enough to argue with you. I don't see that one can argue with that kind of evidence, very much.

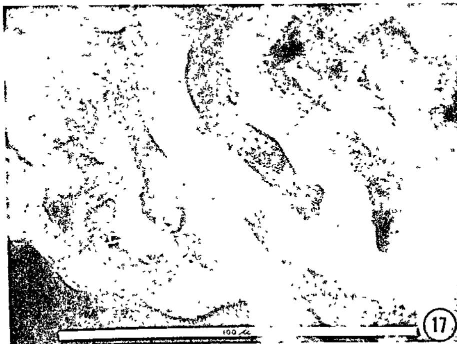
DR. HENRY BARNETT: No one has questioned these data?

DR. HALL: No, not the anatomical evidence for the organization of the glomerular capillaries into lobular networks. However, a critical physiologist friend objects to

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[28] Chambers, Robert, and Zweifach, B. W., "Topography and Function of the Mesenteric Capillary Circulation." Am. Anat., 75: 173-205, 1944.

[29] Trabucco, A., and Marquez, F., "Structure of the Glomerular Tuft," Jour. of Urology, 67: 235-255, 1952.





which are difficult to see in this photograph X1,000

Fig 18 Photomicrograph of part of a lobule of a dissected, latex injected dog glomerulus showing small diameter, anastomosing capillaries arising as branches of larger capillaries The vessel running from top to bottom of picture on the right could be the efferent arteriole The smaller capillaries measure 10μ or less in this region X780



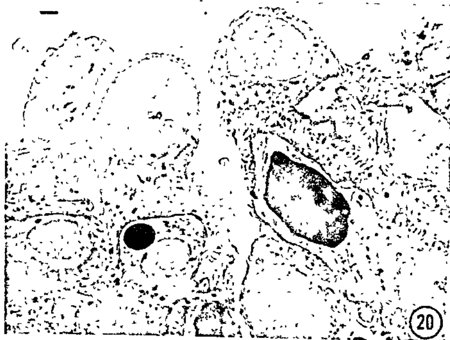


Fig. 19. Photomicrograph of dissected latex injected glomer-
[illegible]
similar to those found in rat and dog glomeruli. In no preparation has it been possible to visualize simple non-branching capillary loops connecting afferent and efferent arterioles. X352



the suggestion that the organization of the capillaries effects 'skimming' of plasma into the small capillaries and that this facilitates filtration. On the other hand, a number of good 'capillary' physiologists and several excellent 'renal' physiologists accept the suggestion as a definite possibility.

CHAIRMAN JANEWAY: It makes renal capillaries very much like capillaries everywhere else in the body, doesn't it?

DR. HALL: I think it very reasonable from a biological point of view that they should be similar.

KR. KURT LANGE. Could we hear a little more about the injection?

DR HALL. Besides what has previously been said I may add that it seems important to stabilize the latex with ammonia, and to use a syringe sufficiently large so that the entire kidney can be filled with the first attempt to force the latex into the kidney There is one trick that we have been using that may be useful. We heparinize animals before using their kidneys for injection, and we have usually used some heparin in flushing the blood out of the human kidneys before attempting to force latex into them The heparin seems to be useful. All injections were made by hand -- nothing elaborate at all.

DR. CONRAD RILEY: Dr Hall, how much opportunity have you had to compare the glomerular capillaries, viewed under the electron microscope, with capillaries elsewhere?

DR HALL: We have had, of course, excellent opportunities to view intertubular capillaries which we usually fail to utilize, but we have obtained some good electron micrographs of a few non-glomerular capillaries. There seems to be nothing comparable to the podocytes covering these capillaries. It appears that much, if not most, of their external surface is in direct contact with tubular basement membranes, with nothing in between basement membrane and endothelial wall. We have a few electron micrographs which seem to demonstrate definite openings or pores in the endothelial cytoplasm of some of the intertubular capillaries: The openings are more difficult to demonstrate clearly and do not appear as numerous as in the glomerular capillaries, but they seem to be there In a recent paper, Dr. Gitlin and our Chairman [30] have presented good evidence that proteins are passing rapidly in quantity through tissue capillaries into the tissue fluids. The presence of endothelial pores in these capillaries, as in the glomerular capillaries, would provide convenient routes through the cytoplasmic barrier for the passage of these proteins, that they find in the extracapillary fluids, and other non-lipoid soluble materials which are known to pass through the capillaries.

CHAIRMAN JANEWAY: There are no cells on the outside of capillaries elsewhere that are comparable to podocytes, even if they don't look like them?

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[30] Gitlin, David, and Janeway, Charles A., "Studies on the Plasma Proteins in the Interstitial Fluid of Muscle," Science, 120: 461-463, 1954.

DR. HALL: Not appearing in our electron micrographs of intertubular capillaries of the kidney in the confined space between two tubules. In the more spacious regions where three tubules come together, there are often extracapillary cells which appear to be connective tissue cells, and in the human kidney collagen fibers in considerable amount may also be found there. But we have yet to find a cell which we could identify as a Rouget cell, or pericyte.

DR. IRVINE PAGE: Dr. Hall, what is the purpose of the endothelial pores?

DR. HALL: Chambers and Zweifach [31] have pointed out that the problem of the passage of non-lipoid soluble materials through the cytoplasm of capillaries is a difficult one. Not having available at that time the recent evidence of electron microscopy on the nature of the capillary endothelium, they postulated that the intercellular cement between cells of the capillary endothelium provided special porous areas where the non-lipoid materials were filtered. But this theory is not useful for glomerular capillaries, since intercellular cement appears to be lacking in these capillaries. Yet, glomerular filtration rates, as we have said, are known to be about 100 times that of other capillaries and non-lipoid soluble materials are known to pass through their walls in quantity. So it seems reasonable to suggest that the numerous holes or pores in the lamina fenestrata of the glomerular capillaries are specializations which function to expose the ultra filtration member, the lamina densa, to the free flow of plasma, by removing the endothelial cytoplasmic barrier.

DR. PAGE: What is the tangential substance lining the capillary -- sort of a membrane inside?

DR. HALL: Yes, a membrane formed by the extension of cortical cytoplasm (ectoplasm) of the endothelial cell inside the capillary away from the region of the nucleus. It is very thin, about 500 A, and specialized by the performations which you have seen.

DR. PAGE: Did you see that at all in regular capillaries?

DR. HALL: Yes, a similar, but generally thicker endothelial lining, at times showing pores, but not frequently. The frequency of the pores seen in some intertubular capillaries is always less than that you have seen today in the glomerular capillaries.

DR. MILTON RAPOPORT: There was some thick material, whose origin was not clear to me.

DR. HALL: All the thickened dense material was lamina densa, or definitive glomerular basement membrane, which we have observed in some human capillaries as appearing very thick. However, it is difficult to know with certainty in electron micrographs whether a membrane has been sectioned perpendicularly or obliquely, and without knowing the angle of the section it is impossible to state accurately how thick or thin the membrane is.

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[31] Chambers, Robert, and Zweifach, B. W., "Intercellular Cement and Capillary Permeability," Physiological Reviews, 27: 436-463, 1947.

DR. DAVID GITLIN: Accepting for the moment these pores in the lamina densa and extra-glomerular capillaries, is there any reconciliation between Pappenheimer's computation of the filtration surface of the capillary wall and estimation of available pore area for filtration as revealed by the electron micrographs?

DR HALL: Yes, there is a reasonably close agreement between the two areas for glomerular capillaries, if one accepts the idea that glomerular filtration takes place only between the pedicels.

DR GITLIN· What about the extra-glomerular capillaries?

DR HALL· We have insufficient data concerning them to make estimates of the total pore area worthwhile

Finally we have arrived at the last illustration, Figure 20. It has been included as preliminary evidence of our current studies on the electron microscopy of developing glomeruli Today's discussion of this phase of the study will be very incomplete, but it may be of some interest. The section shown is from the edge of a developing glomerulus taken from the cortex of the kidney of a 5-day old rat. This and similar sections, especially those taken from even less well developed glomeruli, make it clear that the cells of the visceral layer of Bowman's capsule differentiate pedicels and trabeculae very early in development, long before the lumina of the capillaries become continuous, or circulation established The impression is gained that in these early stages the larger processes (trabeculae) of the podocytes penetrate between the undifferentiated cells which are to organize and to differentiate into capillary endothelium to form capillaries It is possible that the podocytes by extending processes into the mass of undifferentiated cells destined to form capillaries may actually play a role in the formative processes which organize the capillaries into their characteristic patterns.

The evidence of the electron micrographs leaves no opportunity to question, as did the photomicrographs of Guenwald and Popper [32], that the podocytes develop directly from the cells of the visceral layer of Bowman's capsule. Furthermore, we have yet to observe in normal development that cells of the visceral layer slough off into Bowman's space, for each cell of the visceral layer appears to undergo differentiation into a podocyte. The presence of differentiated pedicels and trabeculae on the capillaries before development of a continuous capillary lumen strongly supports the conclusion that podocytes (covering cells) are specialized to facilitate filtration early in the development of glomeruli, even before glomerular circulation is established.

It is noteworthy that Carl Huber [33] about 50 years ago was aware that the glomerulus differnetiates in situ "by proliferation and readjustment of its cells", and stated in his Harvey Lecture [34], that "the renal corpuscle consists of a double-walled

[32] Gruenwald, Peter, and Popper, Hans, "The Histogenesis and Physiology of the Renal Glomerulus in Early Postnatal Life· Histological Examinations," Urology, 43. 452-458, 1940

[33] Huber, G. Carl, "On the Development and Shape of Uriniferous Tubules of Certain of the Higher Mammals," Am. Anat., 4: Supp., pp. 1-98, 1905.

[34] Huber, G Carl, "The Morphology and Structure of the Mammalian Renal Tubule," The Harvey Lectures, pp 100-49, 1909-10.

capsule, the glomerular capsule (Bowman's capsule), usually spoken of as the invaginated end of the renal tubule, though it is not developed by invagination, . . ." Our electron micrographs fully support this statement, since the earliest anlagen of glomeruli often appear as compact masses of cells with no vesicular space. Capsular space early in development, seemingly, is initiated by a flattening of the cells of the parietal layer of Bowman's capsule and the separation of the cells of the parietal and visceral layers of Bowman's capsule from each other. As the renal corpuscle develops, expansion of the diameter of the irregular hollow sphere formed by the parietal layer of Bowman's capsule seems to be more rapid than the increase in diameter of the cellular mass formed by the visceral layer of Bowman's capsule and the cells differentiating into capillaries.

Erythrocytes are frequently observed in these early glomeruli. They seem to appear even before circulation has been established, since the capillaries may have only apparently isolated lumina. This observation that some erythrocytes appear to differentiate in situ in the glomerulus supports the experimental studies of Reinhoff [35], who from observation of the growth of explanted, embryonic chick metanephros in tissue culture, found that "not only epithelial cells, but also endothelial cells and blood cells, differentiate in situ from the mass of undifferentiated cells within the glomerular end of the convoluted tublule which goes to form the glomerulus."

The study of the development of glomeruli with the aid of the electron microscope is only beginning, but the results have stimulated us to continue our efforts in this direction, and to make the suggestion that application of similar methods to other problems in development may be equally, or even more, rewarding. Thank you very much for your time and kind attention

CHAIRMAN JANEWAY: Does anybody have any questions?

DR JACK METCOFF: You left me dangling just a little in terms of what I should substitute for the concept of invagination. Would you clarify that, please?

DR. HALL: The embryologists for a good many years have been studying limb buds, anlagen of relatively undifferentiated cells, which in situ proceed to form some cartilage, some bone, some muscle, etc. It appears that to a certain degree the same thing is happening in the development of the glomerulus. Certainly from careful study of Huber's monograph, which is fully supported by our electron micrographs and by Reinhoff's experimental study, statements which imply that glomeruli develop into double-walled cups because the dilated free ends of developing renal tubules are invaginated by the pushing in of blood vessels, appear to be erroneous. Although it cannot be said to be true of published diagrams, it is true that all actual sections of developing glomeruli in immature kidneys appear to have, in our experience, only very restricted, or no, capsular space, and only in older glomeruli does capsular space appear well established. Over 100 years ago, Gerlach [36] used the example of a man pulling on his 'stocking night-cap' to illustrate his opinion of how invagination covers the glomerular capillaries with a continuous, simple epithelial layer, so that

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[35] Reinhoff, Wm. F., "Development and Growth of the Metanephros or Permanent Kidney in Chick Embryos," Johns Hopkins Hosp. Bull., 33: 392-406, 1922.

[36] Gerlach, Joseph, "Beiträge zur Structurelehre der Niere," Arch. Anat. Physiol. u. wiss. Med., s. 378-387, 1845.

the Malpighian rete mirabile "lies in an invaginated cell layer similar to the intestine in the peritoneum". Even as the 'stocking night-cap' has been outmoded by modern ways of living, so have the erroneous ideas of Gerlach about invagination of the capillaries into the glomerulus and the epithelial nature of the cells covering the glomerular capillaries been completely outmoded by modern means of investigating the glomerulus.

ADDENDUM TO THE DISCUSSION OF THE NATURE OF THE ENDOTHELIAL LINING

In view of the impossibility to determine with certainty how much the presently developed technics of electron microscopy may exaggerate a possibly inherent natural functional porosity, or induce an apparent artificial porosity in the attenuated lining endothelium and definitive basement membrane of the glomerular capillaries, I have decided to avoid further use of the earlier terms "lining net work" and "lamina fenestrata" and suggest that for every usage of the two earlier terms the functionally non-committal and yet anatomically descriptive term, "lamina attenuata" be substituted. Whenever the terms pores, holes, fenestrated, or perforated are used in descriptions of the electron micrographs without the modifiers, apparent or apparently, the modifiers are implied

II. COMPLEMENT PROBLEM

CHAIRMAN MILTON RAPOPORT: To serve as chairman of this conference when the subject matter is serum complement has me at a disadvantage. This is a field beyond my personal experience However, I remember that I was once given a lucrative job as judge at a dog show on the strength of having a connection with a veterinary school -- I was a student helper in the biochemistry laboratory. I got by as a judge of dogs by saying nothing, and watching the other judges. This should serve me now.

Dr. Ralph Wedgwood will introduce the subject of serum complement by defining some of the basic concepts and setting up some points of orientation for the unitiated.

DR RALPH J. WEDGWOOD: Although we have passed the semicentenary of the birth of complement, we are not yet at the stage where we can hold it up, crystalized and purified in a small bottle, and weigh it. Complement, however, is well known and defined instead in terms of function rather than constitution. While these functions include bacteriolytic and bacteriocidal effects, opsonic and virucidal activity, it is best known for its specific capacity to participate and interact in immune phenomena; be it the augmentation of the formation of immune aggregates or the lysis of sensitized red cells by which it is generally measured.

The abbreviation C' is generally used to indicate total lytic complement as measured by the lysis of sensitized red cells. The term sensitized red cells usually refers to washed sheep's red cells to which rabbit anti-sheep-red-cell serum (amboceptor) has been added in an optimal concentration. The amount of amboceptor considered optimal varies in different laboratories, but generally is an amount two to four times the minimum concentration needed to give 50% or 100% lysis of red cells with a slight excess of complement, and which at the same time gives no visible hemagglutination. In most laboratories somewhere between a 1-5% suspension of red cells is used for the measurement

Since magnesium and/or calcium are needed for the function or augmentation of the action of C', and since there is a pH optimum for the system, a buffered isotonic saline solution with added magnesium and calcium is commonly used for all dilutions and suspensions. In addition, since C' is heat labile, stored sera must be kept frozen, optimally at about -70°C., and the dilutions should be carried out in the cold with cold reagents.

Although C' can be estimated by double dilution techniques, it can be measured more precisely by standardized quantitative methods. (Fig. 21) This is the hemolytic curve obtained by repeated measurements on a single human serum. The characteristic S shape, due mainly to the in-homogeneity of the red cell suspension, can be seen. From this curve it can be seen also that the most sensitive and reproducible portion of the titration occurs in the straight line segment which is around 50% hemolysis. The portion of the curve from 10% to about 90% hemolysis can be converted, by use of the von Krogh equation (Fig. 22) and by plotting on logarithmic coordinates, to a quite reproducible straight line.

Many of the abnormal sera that I have titrated have the same slope as the normal, shifted up or down the ordinate. There are some sera in which the slope will differ considerably, particularly sera which have any anti-complementary effect. This does raise the point about the use of a 100% end point rather than the 50% end point. However, with 100% lysis you get what we call the tail off phenomenon, and under such conditions I am not sure it is possible to read 100% lysis as readily or reproducibly as 50% lysis, in spite of the fact that the slope of the line may vary in the latter method.

DR. KURT LANGE: But rather than interpolate from points far apart, they should be replaced with points very close to 50%.

DR WEDGWOOD: By and large when you perform these titrations you usually do get points close to the 50% end point. If the points are too far off, the titrations are repeated to get the points closer. One thing I like about the von Krogh conversion is the fact that you can see the slope This gives you an idea about the type of lytic activity you have in the serum

In the von Krogh conversion the 50% end point is the point where the factor $\frac{\%\ \text{hemolysis}}{100\ -\ \%\ \text{hemolysis}}$ equals unity The reciprocal of the amount of serum indicated on the ordinate by the intercept at this point indicates the hemolytic titer of the serum. In this titration approximately 0.0096 ml. of serum were required to give 50% hemolysis, thus the serum has a hemolytic titer of about 104 units.

C' consists of four known components, all of which are needed for lysis in the hemolytic system. These four components are known as C'1, C'2, C'3 and C'4. They are defined in a negative way by the absence of lytic activity when they are not all present in the serum, and are recognized by their absence in sera treated in various manners.

In guinea-pig serum, the first two components, C'1 and C'2 are heat labile, and can be destroyed by heating serum at 56°C. for 15 to 30 minutes. The third (C'3) and fourth (C'4) components are relatively heat stable. (Table 1)

In human sera, C'3 is heat labile. The first and second components (C'1 and C'2) are defined by the split that occurs in serum proteins dialysed against buffered solutions of phosphate or acetate at pH 5.4 to 5.6, ionic strength 0.02. In such a dialysis the supernate will contain all the C'2, the precipitate the C'1. C'3 and C'4 appear in both fractions, in amounts depending partially on the length of time and the type of

TABLE 1

Material	Treatment	Components present				Material known as	Used to measure
		C' 1	C' 2	C' 3	C' 4		
Whole serum	none	+	+	+	+	C'	
Guinea-pig serum	Heated, 56° for 30 min.			+	+		
Human serum	Heated, 56° for 30 min.				+		
Human serum	Supernate from dialysis at pH 5 6, Ionic Strength 0.02		+	+	+	R1	C' 1
Human serum	Precipitate from dialysis at pH 5.6, Ionic Strength 0.02	+		+	+	R2	C' 2
Human serum	Incubated with Zymosan at 37° C.	+	+		+	R3	C' 3
Human serum	Incubated with hydrazine	+	+	+		R4	C' 4

Note: Small traces of the components "missing" in treated sera may remain after such treatment.

dialysis. The supernate which lacks C' 1 is known as R1, and the precipitate which lacks C' 2 is known as R2.

Neither one of these fractions alone will create lysis of sensitized red cells. However, if the two are recombined so that all four components are once again present in the system, hemolysis will occur. Similarly, if a small amount of an unknown serum containing the missing component is added to either one the hemolytic activity will return. Thus these fractions, R1 and R2, can be used as reagents to measure or demonstrate the presence in unknown samples of the components which they lack.

The third component (C' 3) is specifically inactivated by incubation at 37°C. with Zymosan. Zymosan is the insoluble residue of autolyzed or trypsin digested, boiled, and alcohol extracted yeast. It is essentially insoluble yeast cell wall stroma. Serum treated with Zymosan, lacking C' 3, but containing C' 1, C' 2, and C' 4 is known as

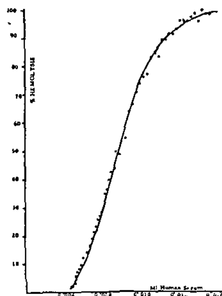


Fig 21

Fig. 21. Hemolytic curve of whole human serum

Fig. 22. Hemolytic curve plotted for the Von Krogh equation

Fig. 23 The relative amounts of R[1] and whole serum that give 50% hemolysis

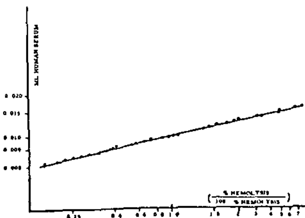


Fig 22

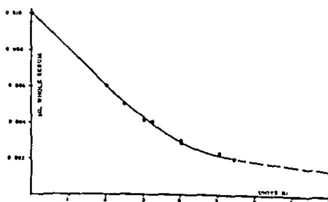


Fig. 23

R3, and can be used in the same manner as R1 and R2 to determine the presence and amount of C' 3 in unknown samples, since on addition of anything containing C' 3 to it, lytic activity will return.

The fourth component (C' 4) is specifically inactivated by incubation with ammonia or hydrazine, and serum so treated, containing C' 1, C' 2 and C' 3 is known as R4, and can be used similarly to measure the amount of C' 4 in unknowns.

The first, second and fourth components (C' 1, C' 2 and C' 4) are the components "fixed" or inactivated in the antigen-antibody reaction. The third component (C' 3) is needed for the final step of lysis in the red cell system. In the human, only the first component (C' 1) has been characterized. It appears to be a euglobulin with a molecular weight of around 160,000.

The reagents (R1, R2, R3 and R4) thus prepared are used to measure the presence of the components of the complement. Such measurement is based on the knowledge that the addition of small amounts of the component missing in the reagent will return the lytic activity which the reagent alone lacks. By definition these reagents must be able to recombine together and produce hemolysis; that is, an R1 plus an R2, or an R1 plus an R3 and so on for the 6 possible combinations must on recombination be lytic. In addition, it must be shown that the reagents are not in themselves inhibitory to the action of complement, they must not be anti-complementary. Of course, by definition, they must not in themselves be lytic in the amounts used for testing.

By and large, R3 and R4 are used as prepared. Since R1 and R2 may be deficient in C' 3 and C' 4 (these components being split between them) in some laboratories these reagents will be "fortified" by the addition of heated guinea-pig serum which contains C' 3 and C' 4 For measurements of the components a constant amount of reagent (R1, R2, R3, or R4) is mixed with varying amounts of the unknown; sensitized red cells are added, and after incubation the tubes are read for hemolysis. Usually a 50% hemolytic end point is used The amount of reagent used is usually two to three times the volume of the serum (from which the reagent was made) which originally gave 100% lysis. This volume of reagent must be known to contain a great excess of all the components except that which is being measured.

Since in such a measurement all components except that being measured are present in excess, the component being measured is assumed to limit the amount of hemolysis produced. Similarly, in whole serum, by the same assumption the total lytic titer should be determined by the titer of the component present in the least amount. This is called the "limiting component." Unfortunately, the titer of the limiting component as measured by the use of reagents is not necessarily the same as the lytic titer of the whole serum. Apparently complement does not add "arithmetically." It is the general experience that the titer of the least or limiting component as measured by reagents is usually two or three times greater than the titer of the serum measured alone.

If any component can be assumed to be truly limiting, then any increase in the concentration of the other three components in the system, so long as the three components are in excess of the limiting component, should produce no increase in the

lytic activity of the system. Conversely, an increase in the concentration of the three components in excess should not alter the amount of the limiting component needed to produce any stated amount of hemolysis. For instance, in the measurement of an unknown serum, an increase in the amount of the reagent used should not decrease the amount of the unknown serum needed to give 50% hemolysis (the usual end point).

These relationships are graphically shown in the next four figures. These were prepared by performing component titrations on the same serum, using the same reagents, but varying the amount of reagent used as well as the amount of serum. In each slide the ordinate indicates the amount of serum used, (in ml), the abscissa the amount of the reagent in "units." The lines and points indicate the relative amounts of each which produced 50% hemolysis.

(Fig. 23) With R1, in the titration of C'1, it is apparent that it is not until you approach 5 units of R1 that the curve begins to level off and an increase in the amount of R1 fails to affect the amount of serum needed to produce 50% lysis. Thus only around 5 units of R1 is there any suggestion of an actual estimate of a really limiting component. This is beyond the range of R1 usually used, because with this amount of R1, the reagent itself may be slightly lytic.

DR. EDWARD FISCHEL: How reproducible is this curve with different reagents, different R1's, and whole serum in the same system?

DR. WEDGWOOD: This is a little difficult to answer. You would probably have to use the same reagent and serum to reproduce a curve exactly like this. The shape of this curve probably depends at least partially on the amount of C'2, C'3 and C'4 you had in the serum from which you made your R1.

(Fig. 24) This shows the same type of titration, but using R2. The curve is much like that for R1, and the conclusions are similar.

(Fig 25) This is the relationship for R3. Interestingly enough, it seems to be almost a straight line. The third component (C'3) does not seem to show any suggestion of becoming "limiting" at all. The amount of hemolysis seems to be able to be increased by increasing the amount of C'1, C'2 and C'4 without changing the amount of C'3 at all, even though C'3 is present in the least concentration in the system.

(Fig. 26) This shows the relationship with R4. This is the only reagent with which there appears an actual limiting function of the least component under normal testing conditions.

DR. LANG: Would you mind explaining this once again?

DR. WEDGWOOD: The points on the lines indicate that when the amount of serum shown on the ordinate and the number of units of reagent on the abscissa were mixed, 50% hemolysis occurred.

DR. JANEWAY: How do you define the units of R4?

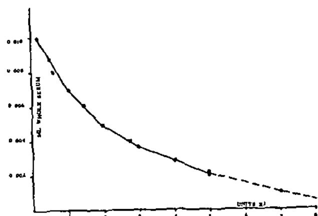


Fig. 24. The relative amounts of R^2 and whole serum giving 50% hemolysis.

Fig. 25. The relative amounts of R^3 and whole serum that give 50% hemolysis.

Fig. 26. The relative amounts of R^4 and whole serum giving 50% hemolysis

Fig 24

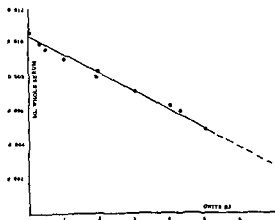


Fig. 25

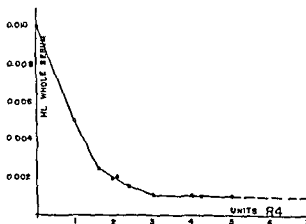


Fig. 26

DR. WEDGWOOD: The definition is volumetric; one unit of R4 (or any of the reagents) is a volume of R4 equivalent to the volume of the serum (from which the R4 was made) which will give 100% hemolysis. The actual volume of the reagent could be indicated on the abscissa instead and you would have exactly the same curve.

What I am trying to show is that if the concentration of C'1, C'2 and C'3 (which by definition is in the serum alone and not in the R4), then an increment in the amount of R4, which contains no C'4, should not produce any increase in lysis; or 50% hemolysis should not occur with any less serum. As shown in Figs. 23, 24 and 25 we found that we could get the same amount of hemolysis (50%) while decreasing the amount of the component which we were measuring by increasing the concentration of the other three components which we were not measuring, but which were in the system.

DR LANG: Does that mean that C'4 is the limiting component in this serum?

DR WEDGWOOD: No In this whole serum, (Fig. 26), using this R4, C'4 did seem to be able to become limiting when enough of the R4 was used. But this was only with 3 or more units of R4. With the other reagents such limitation did not occur even with 5 units of the reagent

DR LANGE Do you actually have a zero point for R4 where you can get hemolysis?

DR WEDGWOOD We have a point where, with no reagent (R4) serum alone will produce 50% hemolysis This is the hemolytic titer of the serum alone. We do not have any points where without serum, the reagent alone will give 50% hemolysis. Beyond 5 units (except in one instance) the line is projected by extrapolation only

DR PHILLIP CALCAGNO· If you had one unit or less of reagent and used 0.2 or 0.02 ml of serum where would you be on the graph?

DR WEDGWOOD. Since one would get more than 50% hemolysis I do not have it on the graph. More than 0.01 ml of this serum produced more than 50% lysis because this serum had a titer of just about 100 units.

DR LANGE. Do I understand correctly that you make C'4 the limiting component in this serum by adding C'1, C'2, and C'3 to it until you come to that point?

DR. WEDGWOOD: What we have done is this -- let's take the R4 --. The R4 contains C'1, C'2 and C'3, but no C'4. The whole serum contained, by definition since it was lytic, all four components. By increasing the amount of R4 we increased the concentration of C'1, C'2 and C'3 in the whole system. There was a point at which even with increase in amounts of these three components, the same amount of serum, which alone contained the C'4, gave 50% lysis. At this point it appears that the C'4 becomes a limiting factor, but this was not true for the R1, R2 and R3 systems in the ranges used.

DR LANGE· Could it be that the R1, R2 and R3 were not completely clean?

DR. WEDGWOOD: The reagents were as clean as we could make them, non-lytic at about 5 units.

DR. LANGE. Aren't you doing it the other way around, instead of doing the regular component titration we all do, if you get, say for C'4 a value of 4 units, and your whole serum has a value of only 1 unit, then you would need 4 times the amount of the others to be added, whatever the limiting component is, in order to come up with C'4 as the limiting factor. Isn't that it?

DR. WEDGWOOD: I am not sure that I was with you.

DR. LANGE: Let's assume that you take a normal serum, and you find in it for C'4 a value of 100 units, and the serum itself has a titer of 25 units, for the whole serum. That means that your C'4 can supply 4 times the amount of this serum which is C'4 before it becomes a limiting factor. Isn't that what you want to say, that you have an excess of C'4 in that?

DR. WEDGWOOD: In Fig 26, by actually measuring the components present in the R4 and serum by the standard techniques for component titrations, at any one point, the C'4 is still the limiting component although decreasing amounts of it give the same amount of lysis -- as is indicated by the slope of the line.

DR. BARNETT. Might not your point be clarified if you showed this for an R1, R2 or R3, rather than the R4, since R4 is the one that did limit?

DR. WEDGWOOD: Let us look at the third component, (C'3) titrated with R3. By the calculations that you suggest, that is, measuring the amounts of the 4 components in the reagent and serum, and adding them together according to the amounts of each in the mixture at any one point we get the following: At the point where 1 unit of R3 mixed with 0.009 ml of serum gives 50% hemolysis there is, by such calculation, 9 units of C'1, 7 units of C'2, 2 units of C'3 and 30 units of C'4; at the point where 5 units of R3 are used, there are 9 units of C'1, 14 units of C'2, 1 unit of C'3 and 74 units of C'4. In each instance in these mixtures, C'3 was limiting; but at one point, there was half as much C'3 as at the other point. Yet even with half the amount of C'3, the same amount (50%) of hemolysis was produced. The concentration of the other 3 components must be thought to have induced this change, where the same amount of lysis could occur in spite of a two-fold decrease in the concentration of the limiting component in the system.

However, I think personally that these additions are hazardous. I do not believe that these absolute amounts -- these arithmetical sums -- are actually meaningful. But, the third component in both cases was by far the least component, and should have been limiting in this system

DR. JOHN A. LUETSCHER, JR.: Dr. Wedgwood, may I ask two elementary questions? Is there an element of dilution in the measurement? Do you bring all the mixtures to the same volume so that increments of one don't change the dilution?

DR. WEDGWOOD: Absolutely.

DR. LUETSCHER: And secondly, are we to suppose that the reagent might not be entirely free of the specific component?

DR. WEDGWOOD: I am quite sure that that is true to some extent.

DR LEUTSCHER: And if that is true, it would account for the continuing slope.

DR. WEDGWOOD: Not entirely.

I wanted to present this in order to suggest that there is considerable difficulty in the precise quantitation of the components of complement. I don't believe that negates in any way the measurement of components. I just think that it is hazardous to attempt to add them together arithmetically. You can see large differences by using some form of internal standardization, by using the same amount of the same reagent to measure a component in the same serum before and after you have done something to it In this manner you can get quite precise results. But when screening multiple sera using different reagents there is considerable difficulty in making any statement as to whether 240 units with one reagent on one day is the same as 240 units with another reagent on another day

This is the only interpretation I would like to suggest at present. Unless you internally standardize your system and use the same reagent throughout, one unit on one day may not react the same as one unit of the same component on the next day. It is unlike total lytic complement ($C'1$) where you can measure the same serum on different days and get the same titer within ± 5%. With different reagents I do not believe that you can get that degree of accuracy.

DR FISCHEL The real problem here is to cope with 8 variables in one system. The term "reagent" implies a constant and rather reproducible standardized system to test an unknown; and yet within the reagent we have at least 3 factors in variable quantities which may affect the total outcome

In addition, each reagent contains anti-complementary factors which have been used to produce the reagents. These are also present in variable quantities. For example, when we produce R4, the excess of ammonia may not be appreciable in crude titrations, but is frequently detectable With these many variables in the reagents, I just got myself in a mathematical maze and gave up.

The other question that Dr Leutscher touched on warrants discussion. I don't think it is valid that by necessarily maintaining constant volume, we are keeping a standardized system.

Dr Otto Plescia in Dr. Heidelberger's laboratory is working on a kinetic system for measuring complement[1]. He varies the concentration of complement and red cells, but maintains a constant ratio of one to the other. There are all sorts of results, even in the total complement picture which we could not appreciate with previous

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[1] Plescia, O. J., Amiran, K., and Heidelberger, M., Reactivity of Complement from Sera of Different Species. Fed. Proc. 13. 508, 1954.

methods. We are forced to recognize that the reagents prepared on any one day are rather unlike the ones of previous days.

DR. LANGE: If you don't standardize them -- but you can standardize them, and get the results within 10% of the substance to be tested.

DR. FISCHEL: If you get constant results, then I would question the sensitivity of the method, because with many variables, some may cancel out others. The unknown being tested is not the determining factor in the titration.

CHAIRMAN RAPOPORT: With small apology, I am going to interrupt this. We have approximately one hour and twenty minutes left for five potential speakers. Dr. Lepow.

DR. IRWIN LEPOW: With this background of discussion, it may appear presumptuous on my part to talk about measurement of complement components in the system we have been dealing with, that is, the mechanism of inactivation of complement by antigen-antibody aggregates.

I fully agree with Dr. Wedgwood that with a single serum pool adding an antigen-antibody aggregate or adding an enzyme, and with a given set of reagents it is possible to measure changes in complement components which at least reflect what is happening in the system. We do not presume that these are quantitative data, but we do feel that we are measuring changes which are of significance in the mechanism of reaction. Under the conditions mentioned, the data are highly reproducible. We became interested in the mechanism of inactivation of complement by antigen-antibody aggregates, not only because the problem is of great intrinsic interest, but also because if we understood the mechanism of complement fixation -- the precise changes which occur from step to step -- we might have a clearer idea of the sequence of events in various allergic and anergic states.

Our work has been with normal human serum. Where we have worked with antigen-antibody aggregates, we have adhered closely to a well defined antigen-antibody system -- pneumococcus S-III -- rabbit anti-S-III. We have used established techniques of immunochemistry, although we are aware of their limitations. We started with some excellent background information from the work of Pillemer, Seifter and Ecker, the Heidelberger group, and others[2]. We knew that when normal human serum is treated with antigen-antibody aggregates, all of the C'2 and C'4 disappears, that we will measure some of the C'1 that was present in the original serum and that we will find essentially all of the C'3[1]. We knew also, again from the work of Pillemer, Seifter and Ecker[3] that it is not possible to inactivate the second and fourth components in a serum which lacks the first component. If one takes R1, as Dr. Wedgwood defined it, and treats it with antigen-antibody aggregates, essentially nothing happens. C'2 and C'4 remain.

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[2] Pillemer, L., Chem. Rev., 33: 1, 1943.
[3] Pillemer, L., Seifter, S., and Ecker, E. E., J. Exper. Med., 75: 421, 1942.

In mulling the problem over before starting any work, it didn't seem likely that a strict adsorption mechanism would apply in this system. We had some information on the kinetics of the reaction which were not compatible with adsorption reactions. The kinetics did suggest an enzymatic reaction. However, enzymatic mechanisms had been postulated from the time of Ehrlich on and hadn't found any experimental verification. During the past two years, Dr Pillemer, Dr. Ratnoff and I, with some very able technical assistance, have tried to get at this problem.

You will recall that we made the observation[4] that when streptokinase is added to human serum, complement is inactivated. Total lytic complement disappears. It was shown that this inactivation of complement was not due to a complement fixation reaction, that is, it was independent of the presence or absence of antibody to streptokinase which could be present in human serum. The inactivation of complement was dependent on the activation of plasminogen to plasmin. Therefore, streptokinase plus plasminogen forms plasmin; plasmin in some manner inactivates complement.

The interesting and startling thing was that the pattern of inactivation of the components of complement by plasmin was the same as we find in antigen-antibody reactions C'2 and C'4 disappear; C'1 is partially inactivated, and C'3 is little or not at all affected.

We thought at this point that we had a mechanism for complement fixation. It had been postulated by Bronfenbrenner[5], Ungar[6], Geiger [6], and others that antigen-antibody aggregates are capable of activating a proteolytic enzyme precursor which resembles plasminogen. The mechanism of complement fixation appeared straightforward· antigen-antibody aggregates convert plasminogen to plasmin; plasmin inactivates the components of complement. We soon found, however, that the situation couldn't be quite so simple. Working with ion-exchanged sera, we found that calcium ions in some manner potentiated this system[8]. In the absence of calcium ions, the inactivation of complement by streptokinase proceeded very poorly. The serum could be returned to its original state by the addition of calcium ions to the serum level of about 2 5 x 10^{-3} M It was the second and fourth components which became resistant to inactivation by plasmin in the absence of calcium ions. C'1 was susceptible to spontaneous inactivation in the absence of calcium ions, so calcium seemed to have some interplay with C'1

Ratnoff[9] and others showed that calcium does not have any effect on the conversion of plasminogen to plasmin by streptokinase. This was confirmed in the course of this work. It was also shown by Ratnoff and others that calcium does not potentiate the action of plasmin on fibrinogen, casein, gelatin, or other substrates. Where then could calcium be playing its role in the inactivation of complement by plasmin?

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[4] Pillemer, L., Ratnoff, O D , Blum, L., and Lepow, I. H., J. Exper. Med., 97: 573, 1953.
[5] Bronfenbrenner, J , Proc. Soc. Exp. Biol. and Med., 12: 3, 1914.
[6] Ungar, G., and Mist, S. H , J. Exper. Med., 90: 39, 1949.
[7] Geiger, W. B., J Immunol., 68. 11, 1952.
[8] Lepow, I. H., Pillemer, L., and Ratnoff, O D., J. Exper. Med., 98: 277, 1953.
[9] Ratnoff, O D., J. Exper. Med., 96: 319, 1952.

We were encouraged that we were dealing with something of importance in the antigen-antibody system by the simultaneous appearance of the work of Levine and Mayer[10] at Hopkins, showing that calcium was involved in the fixation of the components of complement by antigen-antibody aggregates But where was calcium operating in the plasminogen-plasmin-complement system?

We recalled at this point that in an R1, C'2 and C'4 are resistant to inactivation by antigen-antibody aggregates. It seemed of interest to see if this were true for the plasmin system as well, and indeed, we found it to be true[11]. If we took any reagent which lacked or was deficient in the first component, the C'2 and C'4 were resistant to inactivation upon addition of streptokinase or of chloroform-activated bovine plasmin. However, if we were to add to the R1 very small amounts of a C'1 containing reagent, we could restore the system. C'2 and C'4 could once more be inactivated by plasmin or by antigen-antibody aggregates in reactions potentiated by calcium ions.

The type of experimental system used is shown in Figure 27.

We are using R1 as an indicator of factor requirements for the inactivation of C'2 and C'4, either by plasmin or by antigen-antibody aggregates

If we add streptokinase or antigen-antibody aggregates to R1 in the presence or absence of calcium, we get no inactivation of C'2 and C'4. If we add to this system a reagent which contains sufficient C'1, such as R2, R3, R4, RP (a reagent which lacks properdin, the new serum protein that has recently been described[12], or serum which has been heated at 48°C. for 30 minutes, we find complete inactivation of C'2 and C'4 in the presence of calcium. In the absence of calcium, we find partial inactivation, so that calcium is a potentiating factor.

Any reagent deficient in C'1, such as buffer, R1, serum which has been heated at 37°C. for 2 hours, streptokinase-treated serum, or complement-fixed serum -- none of these completes the R1 system if their C'1 titers are sufficiently low. C'2 and C'4 remain resistant to inactivation by plasmin or by antigen-antibody aggregates, both in the presence and absence of calcium ions.

At this point we drew a working hypothesis[11]. We conjectured that the data would fit a scheme such as shown in Figure 28.

In the case of the fibrinolytic system, the addition of streptokinase to human serum converts plasminogen to plasmin Plasmin is then capable of attacking a variety of protein substrates. However, in the complement system, plasmin or antigen-antibody aggregates would be capable of inactivating complement by a mechanism which is

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[10] Levine, L., Cowan, K. M., Osler, A. G., and Mayer, M. M., J. Immunol., 71: 359, 1953; ibid 71: 367, 1953.

[11] Lepow, I. H., Wurz, L., Ratnoff, O. D., and Pillemer, L., J. Immunol., 73: 146, 1954.

[12] Pillemer, L., Blum, L., Lepow, I. H., Ross, O. A., Todd, E. W., and Wardlaw, A. C., Science, 120: 279, 1954.

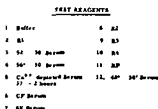


Fig 27 The use of R[1] as an indicator of factor requirements for the inactivation of C' 2 and C' 4 in human serum

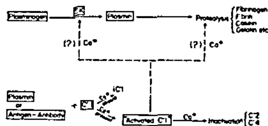


Fig 28. Hypothetical complement activation system

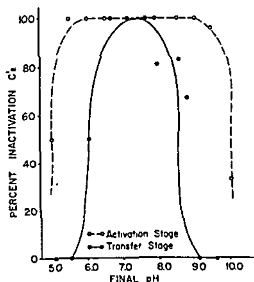


Fig 29 Effect of pH on C' 2 inactivation

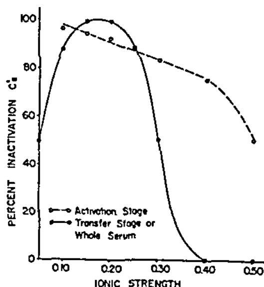


Fig 30. Effect of Ionic strength on C' 2 inactivation

visualized as being essentially the same in both cases: plasmin or antigen-antibody aggregates in some manner react with the first component to form a postulated substance, "activated C'1", which in the presence of calcium ions, is capable of inactivating the second and fourth components.

The mechanism predicts that complement fixation should be a two-stage reaction. We should have a stage involving the reaction of antigen-antibody with C'1. We should have another stage involving the action of the product of the first reaction and leading to the inactivation of the second and fourth components. The second stage should have characteristics of an enzymatic reaction.

We have been able to split the system into two such parts[13] Again, we come back to the basic observation that C'2 and C'4 are not inactivated by antigen-antibody aggregates However, we have found that if an antigen-antibody aggregate is treated with normal serum under conditions of low temperature and short-time, during which complement fixation will not occur, and if the centrifuged aggregate is washed free of occluded serum, and added to an R1, it inactivates the second and fourth components, in reactions potentiated by calcium ions.

In this manner, we distinguish what we call an activation stage, which is the reaction of the antigen-antibody aggregate with a serum factor which we have shown to be C'1 The activation stage will not proceed with reagents which lack C'1.

The second stage we call the transfer stage, which is the transfer of the washed, activated aggregate to an R1 and measuring the disappearance of the second and fourth components

We have studied the characteristics of each of these stages. We have found that the activation stage goes best at low temperatures (0°C.), that it occurs essentially instantaneously, and is insensitive to large changes in pH and ionic strength or to the presence or absence of calcium ions. The pH range here is determined by the stability of C'1, which is somewhere between pH 5.5 and 9.5. On the extreme ends of this range C'1 starts to be inactivated and we no longer activate aggregates.

DR. GITLIN: Is C'1 missing from the whole serum from which you have removed the antigen-antibody precipitate?

DR LEPOW. It is not possible to show with significance the disappearance of C'1 with one treatment of serum with antigen-antibody aggregates at 0°C. for 30 minutes However, if one takes the supernatant serum and recycles the serum on to fresh aggregates three or four times, each aggregate in turn will be activated, and after three or four transfers, one can show a significant drop in C'1 with little effect on other components Thus, C'1 is present in great excess in normal human serum.

The characteristics of the activation stage resemble those of an adsorption reaction low temperature, short time, little pH and ionic strength dependence, and no specific ion involved.

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[13] Lepow, I H., and Pillemer, L., to be published.

On the other hand, the transfer stage has strikingly different characteristics. It is favored by high temperatures (37°C.), although, like complement fixation itself, it will proceed in the cold. It is a time-dependent reaction. It is quite sensitive to pH and ionic strength and it is potentiated by calcium ions. The transfer stage, therefore, has the earmarks of an enzymatic reaction. However, we do not know if it is an enzymatic reaction.

The pH experiments[13] are shown in Fig 29. The final pH is plotted as abscissa, the per cent inactivation of C'2 as ordinate. We get results which are qualitatively similar when we measure C'4, although the two curves are not superimposable. You see that from pH 5.2 or 5.3 on up to pH 9.5 the activation stage is going maximally. It is only when we get out to the extremes where spontaneous inactivation of C' 1 occurs that we find an impairment of the activation stage

The transfer stage, however, is seen to have a fairly sharp pH optimum, around pH 7, which is what one finds for whole serum. At pH 5 5 and 9.0 the transfer stage is completely inhibited.

In contrast, the ionic strength effect is shown in Fig. 30. It is essentially the same experimental system, ionic strength plotted against per cent inactivation of C'2.

You can see that the activation stage is somewhat inhibited by increasing ionic strengths However, it is a slow, gradual effect.

On the other hand, the transfer stage has an extremely sharp ionic strength optimum. The ionic strength optimum, measuring C' 2 disappearance, is 0.15, the ionic strength of serum. Measuring C'4 disappearance, the curve would be displaced toward lower ionic strengths, with the optimum at 0.10 Whether this is significant, I do not know

This, then, is about as far as we have taken the problem. We intend going beyond this to study in more detail the characteristics of each of these reactions. We should like to know the exact nature of the reaction between antigen-antibody and C' 1. Is it just an *adsorption or does some* change take place in C' 1? Is the active material a complex of antigen-antibody-C' 1 or of antigen-antibody- "activated C' 1"? Does something dissociate from the complex which is the active material?

This phase of the problem is in progress, and I hope we can tell you more in a short time. Similarly, we would like to know more about the transfer stage. What are the changes in the molecular structures of C'2 and C'4 that occur when we find them inactivated in this system?

To get a little more speculative, do the altered molecules have any resemblance to the anaphylotoxins mentioned by earlier workers, such as Jobling[14]? Will this provide a clue to what happens in the hypersensitive animal? We would like to know. I hope that by pursuing the work we may get a little bit closer to the answer.

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[14] Jobling, J. W., Peterson, W., and Eggstein, A A., J. Exper. Med., 22: 401, 1915.

DR. DAVID GITLIN: After you have added your antibody complex to the whole serum and spun it off, if you bring that serum back to 37°C., do you get inactivation of C'2 and C'4?

DR. LEPOW: No If serum is treated with antigen-antibody aggregates at 0°C. for one-half hour and the aggregate removed by centrifugation, the complement in the supernatant serum will not be inactivated by incubating it at 37°C.

DR. THOMAS: Presumably, there is not a release of the proteolytic enzyme. Antigen-antibody did not act.

DR. LEPOW: Apparently no significant amount of "dissociation" occurs under these conditions. Otherwise complement would be inactivated when the supernatant serum is incubated at 37°C. We do have preliminary evidence that it is possible to "elute" from activated aggregates at 37°C a soluble product which is capable of inactivating C'2 and C'4. We do not know yet whether we have solubilized a complex or "eluted" a discrete substance. This work is still in progress.

DR. THOMAS: Is there any proteolytic activity in the substance?

DR. LEPOW: We have tested it against casein and gelatin with negative results.

DR FISCHEL Can the amount of C'1 adsorbed by the antigen-antibody aggregate be detected by analysis of the precipitate or is it too minute?

DR. LEPOW: I have attempted this but the results have been within the limits of experimental error.

DR FISCHEL: Even in the multiple precipitate process?

DR. LEPOW: No. All that I have attempted to do is to compare the weight of an activated aggregate with the weight of an unactivated aggregate. I could not detect significant differences. However, my experience with the quantitative techniques have not been of the best, and I really hesitate to place too much reliability on what we have found.

DR. GITLIN: We have tried the same thing.

DR. JACK METCOFF. The details of the transfer system are, of course, very exciting to anyone interested in electrolyte transport, and I am sure you have explored a possible effect of potassium on the calcium sensitive system.

DR. LEPOW: We have treated serums with amberlite IRC-50 in the sodium cycle and then tested the effect of adding back individual cations on the ability of plasmin or of antigen-antibody aggregates to inactivate C'2 and C'4. K^+, Li^+, Mg^{++}, Ba^{++} and Sr^{++} were without effect, while Ca^{++} specifically potentiated the inactivation of C'2 and C'4 in both the plasmin and antigen-antibody systems.

In immune hemolysis, however, Mg^{++} is also involved. Mayer and Levine [10, 15] have shown that a Mg^{++} step follows a Ca^{++} step in the reactions leading to immune hemolysis. We do not know at this time if the Ca^{++} steps of hemolysis and complement fixation are identical. If they are, the Ca^{++} step described by Mayer and Levine would correspond to a summation of the activation and transfer stages which have just been described.

CHAIRMAN RAPOPORT. Dr. Stavitsky has some material he is going to present.

DR. ABRAM STAVITSKY. For many years I have been interested in the relationship of complement to allergic and cytotoxic reactions in vivo. Actually there is a considerable background for this work going back to about 1910. It has been observed repeatedly that there is a marked decrease in the hemolytic complement activity of serum in the course of anaphylaxis [16], serum sickness [17, 18], certain infectious *diseases, including pneumonia* [19], *glomerulonephritis* [20], *and disseminated lupus* erythematosus [21].

It appeared important in the initial approach to this problem to establish beyond a doubt that one could purposely deplete complement in vivo in the course of controlled antigen-antibody reactions. I think you may realize from the sort of things Dr. Lepow talked about that one could visualize other explanations for the decrease of complement that has been observed under these varying conditions.

The work I am going to discuss initially was done with the collaboration of Dr. Ecker at the Institute of Pathology here years ago [22]. These experiments followed a rather simple plan. Rabbits were bled, injected with a protein intravenously and bled again. The hemolytic complement activity of the serum was measured. This same sequence was repeated each time that protein was injected at roughly three or four day intervals, and Table 2 summarizes some of the data that were obtained.

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[15] Mayer, M. M., and Levine, L., J. Immunol., 72: 511, 1954; ibid 72: 516, 1954.
[16] Ecker, E. E., Kovacs, J. and Pillemer, L., Complement in Anaphylactic Shock, Enzymol. 7: 305, 1939.
[17] Paul, B. and Popper, H , Ueber den Komplementsturz im Histaminschock. Ztschr. f. Immunitatsforsch. u exper. Therap. 82: 25, 1934.
[18] Hawn, C. V. Z. and Janeway, C.A., Histologic and Serologic Sequences in Experimental Hypersensitivity, J. Exper. Med. 85: 571, 1947.
[19] Rutstein, D. D. and Walker, W. H., Complement Activity in Pneumonia, J. Clin. Invest. 21: 347, 1942.
[20] Kellett, C. E., Complement Titre in Acute Nephritis, Lancet 2: 1262, 1936.
[21] Wedgwood, R. J. P. and Janeway, C. A., Serum Complement in Children with "Collagen Diseases", Pediatrics 11: 569, 1953.
[22] Stavitsky, A. B., Stavitsky, R. and Ecker, E. E., Loss of Hemolytic Complement Activity and of Granulocytes Following Reinjection of an Antigen into the Rabbit, J. Immunol. 63: 389, 1949.

TABLE 2

CHARACTERISTICS OF COMPLEMENT REDUCTION REACTION

Characteristic	Immuniz. with	Inj. with	Days	Complement 0	1/2	Titer 1 hr.
Specificity	BGG	BSA	0	1.5	1.4	1.7
Persistence	BGG	BGG	1	0.6	5.5	5.5
Cross-reactivity	BGG	PGG	0	0.72	4.8	4.5
Sensitivity	Ea	Ea	0	1.4	1.4	1.3
	Ea	Ea	2	1.4	3.0	2.6
Fixation is by extra-cellular antibody and antigen	BGG	BGG	0	0.76	3.0	2.5
	"	"	14	1.5	3.0	2.5
	"	"	49	1.2	1.1	1.1
	"	"	57	1.0	6.0	2.0
Amount of antigen required	BGG	BGG 200 gammas	0	0.73	1.9	1.45

Complement titer - ml. of 1.10 rabbit serum required to hemolyze 50% of sensitized cells.

The samples marked 0 were collected just prior to the injection of the antigen.

BGG - bovine gamma globulin

BSA - bovine serum albumin

PGG - porcine gamma globulin

Ea - egg albumin

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[19] Rutstein, D. D. and Walker, W. H., Complement Activity in Pneumonia, J. Clin. Invest. 21: 347, 1942.

[20] Kellett, C. E., Complement Titre in Acute Nephritis, Lancet 2: 1262, 1936.

[21] Wedgwood, R. J. P. and Janeway, C. A., Serum Complement in Children with "Collagen Diseases", Pediatrics 11: 569, 1953.

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Fixation is by extra-cellular antibody and antigen	BGG	BGG	0	0 76	3.0	2.5
	"	"	14	1.5	3.0	2.5
	"	"	49	1.2	1.1	1 1
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Ea - egg albumin

We were aware of the possible inaccuracies of the term "complement fixation" so we called the reaction complement reduction. Actually activation reaction might be preferred in the light of recent information.

What was observed was that if one injected bovine gamma globulin, for instance, into an animal, there was no change in the complement the first time the protein was injected. A sample of what occurs the second time is shown here. An animal immunized with bovine gamma globulin was injected with the protein and within half an hour there was a drop in titer. I might say that these are the experimental values obtained. In other words, 0.6 cc of serum to give 50% hemolysis initially, and 5.5 in the later sample, showing a considerable drop in complement activity.

In line with the specific antigen-antibody basis of this reaction was the observation that the injection of bovine serum albumin would not produce a drop in hemolytic complement in an animal immunized with bovine gamma globulin. However, there was evidence of cross reactions between proteins for which we have cross-reactivity data of other types.

Now another interesting point was that this reaction apparently involved extra-cellular antibody and antigen. We were rather excited by the prospect this type of reaction might pick up reactions between antigen and the hypothetical tissue antibody that many people had talked about. However, if one permitted an animal which showed this sort of reaction initially to sit for a number of weeks until its circulating antibody was depleted, reinjection of antigen was not followed by any drop in complement. Of course, following this injection, about a week later the animal had produced antibodies. Again injection of the protein at this time did result in a drop in complement.

The next figure (Fig. 31) shows further evidence for the requirement of circulating antibody for this reaction. We need take only one of these animals here. This was following an injection of 2% bovine gamma globulin into an immunized rabbit. You can see the complement dropped precipitously and to a great extent. The titer returned part way to normal within about an hour and a half, at which time injection of bovine gamma globulin resulted in depletion of complement again. The third time this was done complement was not depleted, but the titer rather returned to normal. Parallel antibody determinations could show antibody was present when each of the first two injections was made, but was no longer present at the time the third injection was made, so it appeared from these experiments that one can show that specific extra-cellular antigen-antibody reactions can result in depletion of complement.

I might say in the light of Dr. Lepow's presentation that when we did reactivation studies with sera in these experiments, C'2 to a great extent and to a lesser extent C'4 were found to have been removed from these sera.

We did run anti-complementary controls on both antigen and antibody since bovine gamma globulin has been *reported anti-complementary* [23] *and we could find no* evidence for this.

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[23] Davis, B. D., Kabat, E. A., Harris, A., and Moore, D. H., The Anti-Complementary Activity of Serum Gamma Globulin, J. Immunol. 49: 2223, 1944.

We are, therefore, left with the conclusion the drop in complement was due to a specific antigen-antibody reaction.

This work has been continued in collaboration with Dr. Heymann and Dr. Hackel. We were not satisfied with the evidence from these experiments that circulating antibody was absolutely required to obtain depletion of complement in vivo. We knew from the other data that the experiments we had done were perhaps not very critical because if one studies in detail the tissues of animals immunized to a variety of proteins after they have once had a titer and had been permitted to sit around the laboratory -- very little antibody is found associated with their tissue. Thus this was not really a critical test of the ability of a tissue antigen-antibody reaction to fix complement.

After all, when one thinks of complement in connection with its possible role in allergic and cytotoxic reactions, one is forced to think of a tissue antigen-antibody reaction. Therefore, we switched to a system in which we knew at least one of the reagents was associated with tissue

This was the system that Smadel [24], Masugi [25], and Heymann and Lund [26] have been studying. That is, the production of nephrotoxic nephrosis in rats by injection of anti-kidney sera.

In this work we have employed both anti-rat kidney rabbit sera and anti-rat kidney duck sera In both cases the sera were injected into the rats

We knew in this case that one of the reagents, the antigen, was definitely associated with tissue, and thus we hoped to be able to get around the objections to the earlier experiments.

Table 3 illustrates the sort of results we have obtained. We took a base line blood sample, then injected the serum and bled the animal. At 30 minutes and 48 hours after the injection, the complement determinations were correlated with determinations of the production of renal disease on a clinical, chemical and pathological basis. Proteinuria, hyperlipemia, and hypertension were produced as well as characteristic pathological changes of renal disease.

If one injects rabbit anti-rat sera, neither renal disease nor a drop in complement occur

With rabbit anti-rat kidney sera various results are obtained One may obtain, as in this case, a considerable depletion of complement with no disease, or one may obtain a drop in complement with renal disease. The titers in Table 3 are the actual

[24] Smadel, J E., Experimental Nephritis in Rats Induced by Injection of Anti-kidney Serum, J Exp. Med. 64: 921, 1936.

[25] Masugi, M., Ueber die experimentelle Glomerulonephritis durch das spezifische Antinierenserum, Beitr pat Anat. 92: 429, 1934

[26] Heymann, W., Lund, H. Z., and Hackel, D. B., The Nephrotic Syndrome in Rats, J. Lab. and Clin. Med. 39: 218, 1952

TABLE 3

Serum Complement Levels and Incidence of Renal Disease in Rats after Injection of Control Rabbit Serum, and Anti-Rat Kidney Serum.

Rat No.	Serum No.	C' at time			Renal disease
		0	30 min.	48 hr.	
1	Normal	.9	.85	.80	0
2		.5	.5	.5	0
3	502 AK	.7	1.9	.5	0
4		1.2	4.1	.7	0
5	506 AK	1.7	4.0	2.0	+
6		1.1	4.0	1.7	+
7	493 AK	1.2	1.4	1 2	0
8		.5	3 0	3.0	+
9		.4	.45	.45	0
10	498 AK	1.7	1 7	1.6	0
11		1.6	3 2	3.1	0
12		1 6	3.4		+

C' -- ml. serum diluted 1:25 required for 50% lysis,
AK -- anti-rat kidney serum.

TABLE 4

Level of Complement after Injection into Rats of Plain Anti-Kidney Serum (AK) or of Anti-Kidney Serum absorbed with Rat Plasma (AK-RP).

Serum No.	C' titer at time		
	0	30 min.	48 hr.
517 AK	.4	3.0	1.8
	.6	3.0	--
	.5	3 4	--
517 AK-RP	.5	3.5	.5
	.5	2.8	.8

C' -- ml. serum diluted 1.25 required for 50% lysis.

TABLE 5

Reduction of Complement after Injection of Anti-Kidney Serum into Bilaterally Nephrectomized Rats

Serum No.	C' titer at time	
	0	30 min.
516 AK	.4	2.5
	1.0	3.0
None	.6	.6
	.4	.4

C' -- ml. serum diluted 1:25 required for 50% lysis.

ml of serum diluted to 25. When one considers the way in which the sera are produced, there is the possibility that the drops in complement are due to antigen-antibody reactions other than a kidney tissue antibody system.

The ones that come to mind first are antibodies to red cells contained in the renal tissue, although the tissues were perfused exhaustively. Also, serum proteins in the rat tissue used for immunization may be antigens; indeed, when one runs very sensitive titrations, in the anti-sera to rat kidney, one does find antibodies to rat serum every time. So in the next experiments we endeavor to get around this by adsorbing the anti-kidney sera with rat erythrocytes on the one hand and rat plasma on the other.

Table 4 shows one experiment in which the anti-kidney serum adsorbed with rat plasma still led to a decrease in complement. Similar results were obtained with kidney antiserum adsorbed with rat cells.

Finally on the basis of the work of Pressman and Cruikshank and Hill and a number of others who showed that kidney anti-sera cross react with a variety of organs in the animal including liver, spleen and lung, we determined what the fate of complement was in bilaterally nephrectomized rats. Table 5 shows these results and indicates the drop in complement in bilaterally nephrectomized rats.

On the basis of these studies, it seems to me that one can conclude only one major thing: insofar as we have been able to exclude the presence of other humoral antigens in the material used for immunization, it appears that tissue antigen-antibody reactions, not necessarily involving kidney because they occurred in the nephrectomized animals, may lead to a decrease in complement

Now, these experiments are not actually incompatible with the postulated role of complement in allergic or cytotoxic reactions We have done anti-complementary controls, and with rare exceptions these sera were not anti-complementary.

Ogawa [27] in 1938 reported that following the injection of rabbit anti-sera, into rats, there was a latent period of a number of days before any evidence of renal disease appeared, and in many of the experiments there appeared to be a very nice correlation between the onset of renal disease and the decrease in serum complement.

At about the same time, or a year or so later, Izumi [28] presented in vitro data showing that in contradistinction to many antisera, duck antisera would not fix complement in-vitro with kidney antigens. Also there were the elegant experiments of Kay [29] with duck anti-kidney sera showing that in contradistinction to the rabbit

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[27] Ogawa, S. and Sato, Y., Ueber das Verhalten des Complement-gehaltes im Serum im Laufe der experimentallen Glomerulonephritis, Tr. Soc. Path , Jap. 28: 212, 1938

[28] Izumi, F , Experimental Studies on Glomerulonephritis, Folia Endocrinol. 16: 48, 1940.

[29] Kay, C. F., Lucchesi, P F. and Rutherford, R. B., An Experimental Investigation of an Immunologic Mechanism as the Cause of Glomerulonephritis. J. Immunol. 42. 369, 1941

anti-rat kidney serum system, where there is evidence of renal destruction in a matter of hours, with the duck system the injection of duck anti-kidney serum into rats and rabbits results in a latent period of many days and possibly even weeks during which nothing very apparent occurs. The explanation that Kay gave was that the initial antibody that was injected combined rather rapidly with the kidney, but did not lead to any damage. Somehow -- and this has always been rather hazy -- some of this duck serum was disseminated to antibody-forming sites and led to the production of antibodies to duck serum. The renal disease resulted only when the host animal produced antibodies to duck serum. The latent period was involved in the production of antibodies to duck serum By appropriate experiments, in which antibody formation was blocked by irradiation or nitrogen mustards, one could show that something in the nature of antibody formation was required for the production of renal disease.

Figure 32 summarizes the experiences with the normal rabbit serum and nephrotoxic serum in rats.

The solid line indicates the complement, the broken line indicates no renal disease developed, plus means renal disease developed.

In general, the controls had no changes, in complement, although in some animals there was a fall in complement with no renal disease; with an early drop in complement, there was a suggestion of early renal disease.

Figure 33 correlates some of the clinical, pathological and complement studies in rats, given duck nephrotoxic serum.

I think it is important to mention at the outset that these animals were bled every one or two days *following the injection of a duck anti-serum. These bleedings were* made by the infraorbital technique described by Dr. Stone [30]. The data represent the results during the initial 10 days on 43 rats. 30% of these animals did not show a drop in complement, but did develop renal disease after a latent period of some 4 or 5 days.

Many of these animals showed a drop in complement at some time later than this, similar to one of the other groups we had below. 26% of these animals showed neither a drop in complement nor renal disease during this period of observation. 17% showed no disease, but a drop in complement after a latent period of somewhere between 5 and 8 days. 12% of the animals interestingly enough, developed a disease with a short latent period of 24 hours or 48 hours, and the drop in complement did not occur until the sixth day. This is analogous to that situation where I believe many of these animals developed a drop in complement many days after the onset of renal disease.

Here is something we were unprepared for on the basis of the previous work. 10% of the animals showed a drop in complement 30 minutes after the injection of anti-serum, but did not develop any renal disease. Only 5% of the animals developed a reaction which was a lot like the one Ogawa described in that there was a nice correlation between the decrease in complement and the onset of renal disease.

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[30] Stone, S., *Method for Obtaining* Venous Blood from the Infraorbital Sinus of the Rat or Mouse. Science 119: 100, 1954.

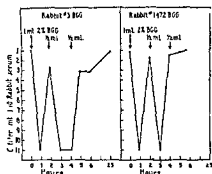


Fig. 31

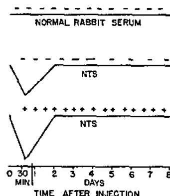


Fig. 32

Fig. 31 Failure of in vivo drop of complement to occur after antibody has been depleted

Fig. 32 Correlation - clinical, pathological and complement studies in rats given normal rabbit serum and NTS

Fig. 33. Correlation - clinical, pathological and complement studies in rats given duck NTS

Fig 34. Complement levels and renal disease in rats given normal duck serum

Fig 35. Correlation - renal disease, complement, and rat anti-duck serum titers in rats given duck NTS

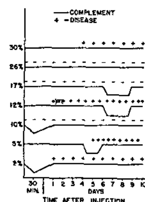


Fig. 33

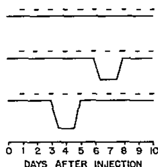


Fig. 34

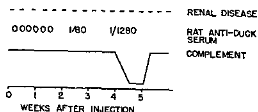


Fig. 35

Similar results were obtained by Drs. Schwab, Janeway and associates who did a number of very elegant experiments in which they administered large amounts of foreign proteins to rabbits, and they found a very nice correlation between the disappearance of the antigen, the appearance of the antibody in the circulation and the onset of renal lesions and other types of lesions [31].

Finally, 2% of the group in which an early drop in complement was observed, had onset of renal disease early, and no latent period in the drop in complement.

DR. LANGE: May I ask if all these results were obtained with the same duck serum?

DR. STAVITSKY: No, they were not obtained with the same duck serum.

DR LANGE Do you get a variety of results with the same duck serum?

DR. STAVITSKY: That is a good point. I do not have the correlation on that.

DR HEYMANN. Usually not. There was usually an uniform pattern with the same duck serum

DR CONRAD RILEY: Dr. Beatrice Seegal, who has been working with this, now has the feeling that it is a quantitative reaction, and that with large enough doses of duck serum, she can get early disease.

DR HEYMANN. That is right That is true in our experience, too.

DR. STAVITSKY: Rats given normal duck serum (Fig. 34) showed neither drop in complement nor renal disease There are animals in which there is an early drop in complement, and a later one which corresponds fairly well to the latent period observed in Figure 31

Figure 35 shows the correlation in a rat between renal disease, complement titers and the antibody [32] of this rat serum to the duck serum, in an animal given nephrotoxic serum but which did not develop renal disease There was a drop in complement about the fourth week at which time there was a considerable antibody titer However, about a week and a half earlier, one could obtain evidence of a slight amount of antibody, whereas, all the earlier samples were negative for antibody. Unfortunately, we do not have a sample in between these two. What this indicates is that one animal may have some antibody at a time far removed from that when the drop in complement occurs.

[31] Schwab, L. and others, Experimental Hypersensitivity in the Rabbit. Effect of Inhibition of Antibody Formation by X-irradiation and Nitrogen Mustards on Histologic and Serologic Sequences and on Behavior of Serum Complement, J. Exper Med 91: 505, 1950

[32] Stavitsky, A. B., Micromethods for the Study of Proteins and Antibodies, I. Procedure and General applications of Hemagglutination and Hemagglutination-Inhibition Reactions with Tannic Acid and Protein-treated Red Blood Cells. J. Immunol. 72: 360, 1954.

I wish we had more data of this latter type. Unfortunately, this is the only experiment in which we had sufficient amounts of material to do parallel antibody and complement titrations.

It is difficult to conclude very much from these data, except that there appears to be a lack of good correlation between the drop in complement, renal disease, pathology, and antibody titer.

Appearance of antibody without appreciable drop in serum complement is not remarkable because Fischel and Benacerraf [33] have shown in studies of antiphylaxis, that small amounts of antigen may cause shock without any drop in complement I don't think these data particularly preclude the participation of complement in these phenomena.

I think one could construct a reasonable explanation for the lack of correlation between the drop in complement and the onset of renal disease. It is conceivable that only very small amounts of antibody and complement might be required to damage tissues, and would be missed by a rather gross titration which depends on picking up reductions in the limiting components of complement

DR. LANGE: In Figure 35, do you have any evidence on how long this high titer of antibody existed -- the 1280 titer you had there?

DR. STAVITSKY: We do not Unfortunately, we have just begun to do these parallel determinations

DR. JANEWAY: There is just one comment I would like to make. I am sure Dr. Stavitsky would be the first one to agree It seems to me it is pretty hopeless to expect good correlations to occur when you are dealing with crude systems like this.

You are putting in a mixture of a large number of antigens together with an antigenic protein group which is also an antibody for something within the animal. So that you have a very, very complex series of proteins I would hazard a guess that the antibody you are measuring in Figure 35 is to a different antigen than the one responsible for the drop in complement One of the things we know very clearly is that serum disease will give just this pattern.

Dr. Longcope showed long ago that there is a rough correlation in serum disease between the appearance of antibody and the symptoms, and presumably complement drops at that time, although he didn't measure it. Provided the dosage isn't too far out of line, in general the globulin components of serum immunize people much more rapidly than albumin components.

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[33] Fischel, E. E. and Benacerraf, B., cited in Fischel, E. E. and Gajdusek, D. C., Serum Complement in Acute Glomerulonephritis and Other Renal Diseases, Amer. J. Med. 12: 190, 1952.

I would hazard a guess that you were measuring precipitans to some of the globulins in the serum, and that the late complement drop you observed was due to the formation of antibody to the albumin components of the serum, since its physiological half-life would appear to be longer than globulin.

DR. STAVITSKY: I have gone back to Ogawa's paper, and the correlation there is not as good as one is led to believe from the summaries of his paper. I think he worked with 8 animals, and of the 8 animals, at least half of them showed renal disease with no appreciable drop in complement.

DR. JANEWAY: I would like to say in relation to the lesions we observed, that bovine gamma globulin does not reproduce nephrotoxic serum nephritis. We used the kidney as a place where the correlations with some patholgic change could be recognized by a pathologist. In our work there was lack of correlation too. It is obvious that correlations are very hard to make if you look outside the kidneys This is a terribly, terribly complicated problem.

DR STAVITSKY: I would like to re-emphasize a point you made I don't think we should, on the basis of these data, conclude that complement plays no role in cytotoxic reactions. The data caution us to look for more precise and careful ways of determining this relationship.

DR HEYMANN. I think the most important finding among these -- would you agree with that, Dr Janeway? -- considering the heterogeneity of our sera, is the considerable number of animals that developed renal disease prior to complement depletion.

DR JANEWAY. I don't know. It seems to me the complement depletion like depletion of any plasma protein is simply a balance between the rate it's produced and the rate it's removed, and you have to have a pretty massive removal of complement to get ahead of what apparently is a pretty rapid synthesis of these components.

DR. STAVITSKY. However, one gets the impression from summaries of the meager literature that there is really an excellent correlation between the onset of renal disease -- I am talking of Ogawa's work -- and the drop in complement. This notion has been perpetuated through the literature until it has almost assumed the status of fact.

DR LANGE Don't you think your disease is entirely different? They have a continuous fall for weeks of complement. That means a production of antibody all the time by the animal itself. But you just give one shot of antibody and get a reaction from it Apparently, the rat, being a very poor antibody former, may be able to form antibodies only to a slight extent, and thereby the mechanism --

DR STAVITSKY: This is possible, but I would like to see the evidence on the latter point as well.

DR FISCHEL I am not intimately familiar with Ogawa's work I have the one illustration he used in his publication, if you wish to see it They injected nephrotoxic

serum, and after several days had a drop in complement with occurrence of albuminuria I thought that might be of interest.

DR. STAVITSKY: There are several animals in the paper in which there is no such correlation.

CHAIRMAN RAPOPORT: We will go on to Dr. Seifter.

DR SAM SEIFTER: It will be my purpose to present some historical data, and to raise some questions about the present work on complement flowing from the knowledge we obtained a number of years ago.

First of all, I should like to continue the discussion concerning the fate of C'2 and C'4 in certain conditions, particularly in infectious disease. We made a very extensive study of the titer of complement and its several components in infectious disease, and found that in those cases in which the overall titer diminished, the decrease was almost invariably due to a decrease in C'2 and/or C'4 titers. In these cases the C'1 titer diminished only sometimes and variably, whereas the C'3 titer was found to remain unaltered. These data, therefore, are in substantial agreement with the experience presented at this conference with regard to the fate of C'2 and C'4 in antigen-antibody reactions, that is, these components are similarly affected both in-vivo and in-vitro

As other workers before us, we also found a number of cases in which the overall complement titer increased, but we found in addition that this increase appeared to be mediated by an increase in the titers of the four components measured

The assay in these studies was based on the determination of that component which limited the expression of overal complement titer, a method somewhat different from that presented by Dr Wedgwood. In our method, we tested diminishing amounts of the serum until the overall complement titer first became zero, and then at that point we fortified with the individual reagent sera in order to determine how much reactivation of the initial fresh serum we would get If we got reactivation, we further diminished the amount of fresh serum, retested with the reagent sera, and continued this process to a point at which we could no longer obtain reactivation. By this procedure of determination of limiting components we were able to distinguish which components apparently diminished or disappeared in certain cases of infectious disease.

Relevant to some of the discussion of complement at this conference, I might also indicate that in some unpublished work on cytotoxic sera, not of the nephrotoxic variety, but specifically the so-called anti-reticulcytotoxic serum, we obtained a significant diminution of complement titers. Dr. Goldblatt had at that time prepared guinea pig anti-reticulocytotoxic rabbit serum, which when injected into guinea pigs caused them to suffer a loss of overall serum complement titer which was due to the primary loss of C'2 and C'4 titers and a smaller diminution of C'1 titer.

In summary of this phase of the work it is apparent that there is a very close relationship between what happens in the test tube and what happens in the intact organism with respect to the titers of complement and its components.

I think it is very profitable to pause and think in simple terms as to what can possibly happen to complement in the body. Principally we should ask the question, "What are the pathways by which a decrease in complement or component titer may occur?"

First, although the term, "inactivation", has sometimes been used interchangeably with "specific fixation", we should consider it entirely conceivable that a non-immune type of inactivation may occur in the organism. We simply have no data in this regard. If such inactivation occurs, C'4 by its very chemical nature would seem to be vulnerable. It is therefore interesting to recall that this component, which by our methods appeared to be that component present in highest titer or at least in titer equal to that of C'1, was the one that seemed to diminish or disappear most readily under a whole variety of circumstances

Secondly, there can be a masking of complement activity by the so-called anti-complementary effects, and I think it pertinent to remark at this conference that whenever one is dealing with the titration of lipemic sera, as in the case of sera obtained from patients with the nephrotic syndrome, it is necessary in each instance to run a control test for anti-complementary activity It is not sufficient to extrapolate from the results with one serum to all other sera of similar type.

Thirdly, there can be specific immune fixation of the type that we have seen in test tube situations The presumptive evidence is strong, judging from the components which "disappear" in certain infectious diseases, that at least some fixation of the specific immune variety may occur in the organism as such It may not be fixation to an antigen-antibody aggregate, but utilization in another type of immune reaction

Fourthly, there can be interference with the production of complement, as Dr. Janeway remarked, and finally, and perhaps least likely, loss by excretion in the urine.

In this latter connection I want to mention the work we did on the excretion of the complement components in urine, work which deals with the subject of the nephrotic syndrome I must state that our object in such a study at that time was somewhat different from the implications we read into it nine or ten years later.

At the time of the work there was still some question as to whether the proteins excreted in the urine in the course of the nephrotic syndrome were "normal, physiological" proteins, and our main interest was to have a naturally tagged protein In other words, we wanted to study the excretion of a protein with some biological activity, and for this purpose we chose complement in the first instance and the isohemagglutinins secondarily Our results showed that while complement as a whole did not appear in the urine, several of the discrete components were excreted in significant amounts by nephrotic individuals It was also shown that the isohemagglutinins could appear in the urine We felt, therefore, that this was a demonstration that proteins with specific physiological activity in the normal sense appeared in the urine in the course of the nephrotic syndrome

We were also interested in the relationship of this loss of complement components to the matter of resistance to infection We stated that the urinary loss of complement

was perhaps one pathway, among others, by which resistance to infection was diminished in individuals with the nephrotic syndrome.

In the paper we published on this study we did state that we observed some diminution of complement titers in nephrotic individuals, and in some instances the change in serum titer was concomitant with the loss of complement to the urine. However, we were very wary concerning the significance of the loss of serum complement titer since we did have some trouble with regard to anti-complementary effects presumably due to lipemia. Since at this time I left the laboratory, and the matter was pursued no further, I cannot say whether the anti-complementariness of sera from such individuals is a general thing; but we did conclude at the time that the amount of complement found intact in the urine was not sufficient in itself to account for a significant lowering of the serum complement titer.

Briefly I should like to return to a point relevant to the discussions of Drs. Wedgwood and Lepow. It was mentioned that in the case of the human complement system as well as the guinea pig, immune hemolysis occurs with the "fixation" of C'2 and C'4, and that no such "fixation" occurs without the presence of C'1. That this importance of C'1 for the fixation of the other two components is more general, is implicit in results we obtained with the fixation of complement and complement reagent sera to a system of Vibrio comma and its specific antiserum. Here too C'2 and C'4 were fixed, but again only in the presence of C' 1 However, this dependence has only been demonstrated in such experiments taking place outside of the animal organism.

Therefore, I think it would be instructive for Dr. Lange to extend his perfusion experiments, which he will undoubtedly describe in detail later, to include the circulation of specifically inactivated complements (reagent sera) through the kidney. Dr. Lange finds that the perfusion of the kidney with fresh serum and nephrotoxic serum results in the removal of complement components, and evidently an antigen-antibody reaction is responsible for the result. It should be possible to determine in the isolated kidney, as it has in the test tube, whether complement components will be fixed from sera lacking in C'1 Should C'1 prove to be necessary for the fixation of C'2 and C'4 in this system, it could be interpreted as additional presumptive evidence that an antigen-antibody reaction is responsible for the removal of the individual components.

Finally, I think that this discussion should not conclude without recognizing the fact that we are now attributing a new activity to complement, that is, we are saying that complement may participate in cytotoxic or auto-cytotoxic activities. The first new activity ascribed to complement for a long time turns out to be a destructive one. We have always been accustomed to think of the complement system as being protective in the immunologic sense, and now we are confronted with the concept that it, like antibody, under particular circumstances may be destructive to the organism elaborating it.

CHAIRMAN RAPOPORT: We will continue with Dr. Lange.

DR. LANGE: The fact that complement is lowered in the human in acute and subacute glomerulonephritis and in the nephrotic syndrome has raised the question whether this lowering is due to the use of large amounts of complement in an antigen-

antibody reaction or whether it is not dependent on such a reaction but only represents another side effect of the disease.

For that reason we started a group of animal experiments which, although they may not be directly transferable to the human disease, may throw some light on it.

In our first group of experiments we isolated rat kidneys, ligated the ureters and perfused them from artery to vein with fresh rat serum [34]. The kidneys were perfused with recirculation by a small pump, using approximately 6 cc of rat serum in the recirculation. The reservoir which is included in the set-up permits the adding of nephrotoxic sera and the withdrawal of samples at any desirable time.

A sample of the perfusing serum was taken for complement determination before perfusion through the kidney. Another sample was taken after perfusion through the kidney, but before the addition of nephrotoxic serum. There is an initial drop of complement titer in all perfusions which may be due to adsorption of some of the complement on the surface of the glomerular apparatus which was previously washed free of serum by saline perfusion. After this initial drop, however, the complement titers stay constant if nephrotoxic sera are not added. If one splits the complement into its fractions before and after perfusion, one finds that the initial fall in titer is due to a lowering of all components, thus giving the impression that we are dealing with a plain adsorption phenomenon.

When subsequently 0.6 ml of anti-rat-kidney rabbit serum are added to the perfusing rat serum, the complement titer is not influenced until this mixture has passed through the kidney. After one, two or three passages through the kidney, however, the complement titer goes down to zero This experiment was repeated in 18 different perfusion experiments with an identical result in each of them. While in one instance only one passage through the kidney may be necessary to remove all complement activity, in other instances up to three passages may be required.

If one uses anti-rat-kidney duck serum, the fall in complement titer does not occur. This was demonstrated in 5 experiments using 3 different sera We are grateful to Dr Heyman who sent us some of his anti-rat-kidney duck sera which showed an identical behavior with the sera of our own production. If one adds, in such an experiment, anti-rat-kidney rabbit serum at a later time, a precipitous fall in complement titer occurs, indicating that the preparation in itself was not at fault. A similar behavior of anti-rat-kidney duck serum was demonstrated by Izumi who was able to show that in vitro duck immune sera do not bind complement in an antigen-antibody reaction with mammalian antigens. When plain rabbit serum is added to the perfusing rat serum, a fall in complement titer does not occur.

Adding Cortisone to the perfusate does not prevent the fall in complement. All anti-sera used were tested for anticomplementary action and only those were employed which did not show an anticomplementary effect. When the nephrotoxic serum is added in small increments, complement also falls in increments in a fairly straight line. Perfusion of rat liver with rat serum to which nephrotoxic sera were added also led

[34] Lange, K and Wenk, E J , Am. J. Med. Sciences, 228: 454, 1954.

to a fall in complement titer. However, the amount of complement removed by the liver on a weight for weight basis is much smaller than the amount removed by the kidney, indicating, as Dr. Heymann and Dr. Stavitsky have mentioned, and as Pressman has stressed before, that this reaction is not confined exclusively to the kidney, but seems to occur in the entire vascular tree. From these experiments it appears that the fall in complement is due to an antigen-antibody reaction, since in this set-up complement cannot possibly escape into the urine.

When the sera, after perfusion with fall in complement, were analyzed for individual complement components, it was found that mainly component C'2 is removed, while component C'4 is slightly depressed, and components C'1 and C'3 are not concerned at all. In these experiments, the ureters were ligated right at the kidney pelvis, so that a loss of proteins into the urine can be safely excluded. The fact remains, then, that during perfusion of rat kidneys with nephrotoxic sera which in the intact animal, produce a nephrotic syndrome with great regularity, complement is removed from the perfusate rapidly and in large amounts. The main component removed, the limiting component, is C'2

We now turn to the behavior of complement in experimental nephritis in the intact animal. Dr. Stavitsky and Dr. Heymann have shown that in the nephrotic syndrome produced in the rat by anti-rat-kidney rabbit sera, complement falls immediately, but rises quickly thereafter, to return to normal within 20 hours. These findings confirm the findings of Pfeiffer and his collaborators [35]. Our own experiments with this type of experimental nephritis is shown in Figure 36A, in which a group of 8 rats is described before and after the injection of nephrotoxic rabbit serum. The C' titer falls precipitously, but in 24 hours it is back to normal, even over-shooting it, and then returns to the previous value. Some animals went down to a complement level of zero and died. Eight days after the injection, we studied again the complement levels in those animals which had survived, to see whether there was a secondary response, but there was no fall in any one of them. Values taken 2 weeks later again were well within the normal range The controls shown in the lower part of the table show a good correspondence of values taken at different time intervals, so that we can exclude spontaneous variations as the cause of the fall in complement in the treated group. We thus have to assume that in contrast to the human disease we produce in the rat disease one short-lived injury to the kidney and then the severely damaged kidney goes on showing clinical sequelae of this damage.

The behavior is entirely different in the rabbit when given anti-rabbit-kidney duck serum as it was originally investigated by Masugi and further studied by Ogawa and Sato (Fig. 36B). In the rabbit, complement does not fall immediately after the injection of nephrotoxic duck serum. Irrespective of the dose given, such a fall cannot be observed for 5-7 days. On the fifth to seventh day, 29 rabbits thus studied showed a severe albuminuria and a concomitant fall in serum complement. The fall in complement even antecedes by a few hours the appearance of albuminuria and even animals which become completely anuric show a precipitous fall in complement. Since the anuric (catheterized) animal does not lose any protein into the urine, but the complement falls nevertheless, this indicates again that the fall in complement cannot be blamed

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[35] Pfeiffer et al., Ztschr. ges. exp. Med. 122: 446, 1954.

INFLUENCE OF ANTIRATKIDNEY RABBIT SERUM (ARKR)
ON THE COMPLEMENT TITER OF RATS

Animal No	C' Before Injection	C' 1 Hour After Injection of 0 8 ml. ARKR	C' 24 Hours After Injection	C' 8 Days After Injection
1	1.7	1.0	2.9	1.9
2	3 6	2.0	D	
3	2 0	0.77	2.0	1 8
4	2 0	0.12	D	
5	3 5	1 3	D	
6	1 1	0	D	
7	1 1	0	1.1	1.2
8	1 6	0	1.5	1.7
		Controls		
9	1.0	1 1	1.2	
10	1 9	1.7	1.9	
11	2 0	1 7	2.0	

D - Died

Fig. 36A. Effect of antirat kidney rabbit serum on complement titer of rats

Figure 36B Effect of Anti-Rabbit Kidney Duck Serum on Complement Titer of a Male Albino Rabbit, 3390 gm

Day of exper	Procedure	Complement units	URINE Volume	Prot 24/hrs.g	RBC HPF	Casts	BLOOD BUN	Cholest.
-1		1 4	90	--	--	--	12	134
0	Antirabbit kidney duck serum 5cc i v		110	--	--	--		
1	"	1 2	40	--	--	--		
2	"	1 9	20	--	--	--		
3		1.4	45	--	--	--		
4		2 7	60	--	--	--		
5		0 77	15	0 5	15	occ		
6		0 66	45	0 5	15	--		
7		0 58	40	0 43	30	--	122	178
			33	0 3	50	--		
9		0 52	35	0 6	40	many		
10			18	0 7	30	many		
11			35	0 8	45	many		
12		0 93						
13		0 99	100	0.2	20	few	190	162
15		0 87	60	0 8	25	occ.		
16			25	0.8	10	--		
17			60	0 2	--	--		
18			15	0.05	--	--		
19			80	0 3	occ	--		
20		0 92	110	0 2	7	--	76	273
21			130	0 2	7	--		
22			170	0 3	9	--		
23			90	0.4	15	--		
25		0 14	140	0.8	occ	--		
27		0.51	180	0 9	6	--		
29			250	0.8	20	--	49	283
33		0.13	210	1.2	--	--	20	
34	FOUND DEAD AUTOPSY PERFORMED							

Fig 36B *Effect of antirabbit kidney duck serum on complement titer of rabbits.*

on loss of complement or one of its components into the urine. The severity of the disease is approximately proportionate to the amount of nephrotoxic duck serum initially injected. The highest mortality rate is in the group with the highest dosage, and the fastest recovery is in the group with the lowest dose. I wish to stress again that, even with the greatest doses applied by us, we were not able to shorten the minimum interval of 5 days between the injection of the nephrotoxic serum and the immunologic and clinical appearance of disease.

The NPN rises in the majority of animals with the onset of disease, and also roughly in proportion to the amount of nephrotoxic serum given. Even after the return of complement to normal a moderate or mild degree of albuminuria may persist for several days or weeks, a feature which is similar to the human disease. A number of our rabbits developed a nephrotic syndrome with fluid accumulation in the body cavities, severe constant albuminuria and an elevated serum cholesterol. We feel that this type of disease simulates more closely the human disease. It is characterized by a time lag of approximately one week between initial injury and occurrence of disease. The same time lag is required for the fall in complement. In addition complement stays low in this type of disease until the animal either dies or heals with or without defect.

The analysis for individual complement components of the sera of such rabbits with low serum complement reveals that C'2 becomes the limiting factor, while C'4 is either not at all or only very slightly concerned. C'1 and C'3 remain unaltered. Morphologically, these kidneys often showed a flea-bitten appearance, or the picture of the large white kidney with poor definition of pyramids and occasionally with a capsule which stripped poorly. Histological studies of these kidneys are under way.

I now turn to our observations on serum complement in patients with acute glomerulonephritis and the nephrotic syndrome. As you know, we found that in all cases of acute human glomerulonephritis, and most cases with the nephrotic syndrome, serum complement levels are low. In 166 control cases (198 determinations), the range of complement was between 1.1 and 3.0 units. The high values represent mostly post-infectious stages where complement is usually high as a rebound phenomenon described previously. Forty cases of acute glomerulonephritis with 171 determinations showed values between 0 and 1 with an average of 0.32 units compared with an average of 1.78 units for the control group. In the nephrotic syndrome the values are somewhat higher than in the acute glomerulonephritics, but clearly subnormal, the average being 0.64 units (29 cases, 225 determinations; 9 adults, 20 children). In the nephrotic group you find a few cases with normal complement values. Such behavior is understandable. If the reaction is only mild, a normal supply of complement may suffice to cover the needs of the antigen-antibody reaction without a fall in the overall level of complement. While this behavior of complement in nephritis and in the nephrotic syndrome has been confirmed by numerous investigators, the reasons for this fall remain under discussion.

When a case of acute glomerulonephritis heals, complement rises, and approximately at the time when complement returns to normal, clinical signs and symptoms disappear. There are some deviations from this rule insofar as complement may return to normal somewhat faster than the disappearance of the clinical signs, and vice versa.

In the nephrotic syndrome complement is usually low. It returns to normal just prior to spontaneous remissions. Thirteen instances of spontaneous diuresis were observed, 11 of which were preceded by high fever due to intercurrent infections 6 to 10 days prior to the diuresis We feel that this diuresis may be due to a stress phenomenon with ACTH output, documented in these 11 instances by a fall of eosinophiles to 0 In 2 instances we also examined the 17-Ketosteroids and found them to be elevated during the episode of fever We believe, therefore, that the spontaneous diuresis is due to ACTH output during the stress period. Approximately 50% of our cases diuresing spontaneously or subsequent to ACTH or Cortisone medication, showed a fall in 24 hour urinary protein output when diuresis occurred. The rest, however, did not show such a fall, and the osmotic pressure of the plasma measured directly, did not change. The question has been brought up whether the fall in complement in the nephrotic syndrome could not be due to a loss of complement or one of its components into the urine. Several years ago Dr Seifter had shown that the amount of complement components found in the urine was too small to explain the fall in serum complement. In addition, we found that in Kimmelstiel-Wilson's disease, with similar degrees of proteinuria, similar or larger amounts of complement components are lost in the urine, but the serum complement levels, nevertheless, stay perfectly normal. The same held true in one case of renal amyloidosis which we observed. As we have demonstrated previously, ACTH and Cortisone given for 7 to 10 days to individuals with the nephrotic syndrome lead to a rise in serum complement shortly before the occurrence of diuresis. In our opinion, this is the consequence of a suppression of antibody formation by the steroids. When such cases diurese, the amount of individual complement components lost into the urine does not vary materially from the amount lost prior to diuresis. We do not find any definite relation between the complement component excreted into the urine, and the type of disease. Moreover, if one determines the maximal excretion of complement components in 24 hours, and assumes that the loss of one complement component is as good as if the whole complement were lost, one finds that the amount of complement excreted in 24 hours is too small to account for the lowered serum complement values.

If one analyzes the sera of patients with acute glomerulonephritis and the nephrotic syndrome for individual complement components, a peculiarly constant observation is made. Sera of patients with acute glomerulonephritis with lowered complement titer show that $C'4$ is the limiting component. Only when the overall titer is down to 0 by a reduction of $C'4$ to 0, a lowering of other complement components, especially $C'2$, can be noted also.

In contrast to this, 10 cases with the nephrotic syndrome all showed a lowering of serum complement by a predominant lowering of $C'2$. In this instance $C'4$ is only mildly decreased. These patterns are so constant that we can now blindly analyze sera and state whether they came from nephritics or nephrotics, depending on whether $C'4$ or $C'2$ is the limiting component.

The reasons for this difference are not clear at present. As I mentioned before, the lowering of complement values in experimental nephritis in the intact animal as well as in the isolated kidney, was always found to be due to $C'2$ as the limiting component. This behavior resembles the pattern found in the nephrotic syndrome of humans if one wishes to draw a parallel between the complement in humans and in experimental animals.

In order to see what role the liver plays in complement production, we analyzed, with Dr. E. E. Mandel of the U. S. Public Health Service, the sera of 15 cases of viral hepatitis for serum complement content. Of these 15 cases, 4 were due to homalogous serum sickness. All 15 cases had high complement titers. Fifteen cases of severe cirrhosis of the liver were examined at different stages of the disease and all of them had normal complement titers. In some of these patients, the cirrhosis was quite advanced, and they were in hepatic coma. The sera of 7 cases of acute cholecystitis based on choleslithiasis were studied and found to have very high levels during the inflammatory stage and normal levels after the disease had subsided. These findings seem to indicate that the liver apparently does not have too much to do with the formation of complement. Three cases in which at least half of the liver was removed for hepatomas were also found to have normal or high complement values.

DR. CALCAGNO. What happens to the C'2 component after therapy, and with partial therapy? I notice of the number of patients you studied, 4 were adults. Does the same thing happen to them as happens to children?

DR. LANGE: What the pediatrician likes to think of as pure nephrosis in the child has the same complement response as the nephrotic syndrome in the adult.

We have several cases we followed during therapy. The limiting component, C'2, comes up and overall titer comes up with it with diuresis

DR BARNETT In your perfusion experiment, did you measure the concentration of any other substances in blood except complement?

DR LANGE No.

DR BARNETT. It would be interesting to know whether or not protein changed even though the ureters were tied off.

DR. LANGE: It hasn't been done.

DR. MAX MILLER· What does complement do in cases of chronic glomerulonephritis without edema?

DR LANGE. I don't like the term chronic nephritis. It is only subacute nephritis, and in subacute, complement stays low. We have cases that stayed low in C'4 and now are pre-uremic, uremic or have died after several years with low complement. Others may have a mild albuminuria, but have lost much of their glomerular tissue

DR. ROBERT TAYLOR· I don't think there is a low complement titer in chronic nephritis. They have what Dr Heymann has in his rats. Such patients have no "itis" any more, no inflammatory fresh lesion They are left with a defect which may, with increasing age and scarring, finally lead to uremia. These are not cases of uremia in a few years, but cases that die 20 or 30 years later, if they die at all, from renal disease.

DR. HEYMANN: Dr. Lange, do you think that there is a relation to the differing degree of proteinuria in "pure" nephrotics and acute nephritics in that the ratio of albumin excretion in the nephrotics is higher than that of the acute nephritics. As far as our experiences are concerned, we have followed accurately 22 nephrotic children with daily quantitative urine protein determinations We had 4 children who did not diurese, and no decrease in proteinuria occurred. Eighteen diuresed, and in all of them preceding diuresis, decrease of proteinuria occurred.

DR LANGE: As to the first question, we have analyzed acute nephritics for albuminuria and have not found an increased output of C'4.

I don't know whether the C'4 is lost somewhere else, but we could not show it to be lost in the urine. Whether it was somewhat masked by some other process in the urine, I don't know.

We have routinely analyzed 24 hour urines of children with the nephrotic syndrome prior to diuresis, and after diuresis. Around 50% of them do not show an immediate reduction in proteinuria.

DR. SEIFTER: I should like to comment on the subject of complement in the urinary proteins. I think it is very important to remember that you cannot quantitate complement in the urine; for one thing, the urine is stored in the bladder usually under acid conditions but occasionally under alkaline conditions. All that you can do is give an idea of what components there are in the urine, and set a lower limit for the amount of each component present. I think it incorrect to try to assume from what one finds in the urine the amount that has actually disappeared from the blood.

We had one case in which the pH of the urine was on the alkaline side, and with ammonia appearing in urine under such conditions one may expect that some or all of the C'4 will disappear, and we would not be cognizant of this component in our analysis. So going backwards from the amount found in the urine to the amount which should have disappeared from the blood does not give the correct answer. In my earlier discussion the only reason I said that I didn't believe that the urinary excretion of complement could be made to account entirely for the decrease of complement titer in the serum was because we didn't consistently find such a decrease, particularly since we were worried that we hadn't sufficiently ruled out the presence of anti-complementary substances However, I do not deny that a decrease in complement activity in the sera of nephrotic individuals may occur.

It is interesting to remark that if one were willing to accept the idea that perhaps the removal of complement to the urine, even though it may be inactivated before detection, may cause a significant lowering of the serum complement, one might then account for the difference of complement pattern in the serum of the acute glomerulonephritic as opposed to that of the nephrotic.

In acute glomerulonephritis, the picture with respect to C'4 looks very much like that obtained in infectious disease, particularly in some of the streptococcal infections. In the case of the nephrotic syndrome the serum pattern could then be consistent with the loss of the particular components to the urine.

I am not suggesting that this is the actual mechanism of the difference, but I am saying that it is something which has to be considered.

DR. WEDGWOOD. In your slides of the rabbits injected with duck anti-rabbit-kidney serum, I notice something that we saw in our work following the injection of bovine serum gamma globulin into rabbits. Following the initial injection in some of your rabbits, and in some of ours, prior to the fall in complement, there is a slight rise in the C' titer. We also found, as I remember, a slight rise in serum complement titers of animals given ACTH alone. We have kept quiet about this because we had doubts about what it meant. But now I wonder. If the stress induced by ACTH injection, or the injection of a foreign protein can produce a rise in C'; whether the stress of, for instance, fever, prior to diuresis in a nephrotic might not be one explanation of the increase in C' seen in nephrotics at such times.

Secondly, to re-echo what Dr. Seifter has said, destruction of C' in urine has been measured Complement may disappear quite fast if one incubates it at 37°C in urine, and $C'2$ and $C'4$ are the components destroyed most rapidly. One wonders whether the same thing might not occur in the bladder. This would make the actual urinary excretion of $C'2$ and $C'4$ a very difficult thing to measure.

DR LANGE: In order to overcome that, Dr. Seifter, we have done the following thing: We have taken normal urines and added human serum with known complement value. Recovery experiments gave 85% recovery which we thought was fair enough. The rise in complement which you mentioned we have noticed also I think there is a mild, immediate antigen-antibody reaction after infectious diseases.

CHAIRMAN RAPOPORT. Dr. Fischel.

DR. FISCHEL: One of the advantages of coming at the end of a long series of very erudite discussions is that I have very little new to offer which might compound the confusion

As with many in the field, our interest in complement evolved from its intriguing manifestations during a known allergic reaction such as serum sickness We attempted to apply this to document the process of allergy in rheumatic fever

Complement itself has always been defined essentially in immunological terms by the gross process of immune hemolysis, bacteriolysis, or opsonic activity, and it has always been associated with the study of infectious disease It has been, therefore, very strikingly brought home to us that it is not alone used in the body economy in infectious diseases In sterile conditions such as myocardial infarction, we find a very dramatic, very precipitous, rise in serum complement An increase in complement also occurs in pulmonary infarct, Hodgkin's Disease, etc In short, any trauma or inflammation associated with the so-called acute phase reaction, such as conditions that usually result in fever or leukocytosis or sedimentation rate rise, will usually, although not constantly, result in a rise in complement.

Dr. Janeway touched on a very important point this morning in our interpretations, namely, that there is a dynamic state, a turnover of complement. It is being produced continuously. It is being used continuously, and I would hazard the guess that it is not being used in immune reactions as much as in some other reparative or non-specific process in the body. Therefore, when we find that there is no parallel between increased proteinuria and a decrease in serum complement, the interpretation should not assume that there is a constant level of complement in the body. There are mechanisms of production which can be stimulated and certainly are stimulated.

Now, suppose we review some of our resurrected data because they may emphasize some of the problems that we are particularly interested in at this time.

The first one concerns methodology. I shall not belabor that point. We have had enough discussion on it so that all of you are aware that there are many gaps, many things to be desired in our measurement, both of total complement and in complement components. We have been content to use the method of Mayer, Osler, Bier and Heidelberger [36] for measuring total complement

Despite the problem of method, once we have an arbitrary system, it is amazing how in the measurement of total complement the reproducibility of the system is a very beautiful one, and is within the limits of most biological systems.

Figure 37 applies particularly to the maintenance of normal values in adults and children. We have our rather arbitrary method of a certain number of cells, in a certain volume, buffered solution with fixed ionic strength and added magnesium and calcium. This illustration represents some 50 cases, but we now have about 100, all falling within a rather narrow range between 30 and 45 units. The mean is 38 ± 4 units ± 1 sigma).

Dr. David Earle, when he was at New York University, and Dr. Wedgwood when he was in Boston, wrote me that they obtain slightly higher values for normals. I don't know what the differences are: in the sheep that provide the red cells; in the water we use, or what the variable is. The values are not much different. They are shifted a little higher. Is that right, Dr. Wedgwood? Nevertheless, there is a remarkable constancy in the normal individual.

Figure 37 also shows some of our results in rheumatic fever [37] At the time this was done, an increase in serum complement was not a well appreciated phenomenon. Most people, influenced by the emphasis on complement fixation in the test tube, as in the Bordet-Gengou phenomenon and the Wasserman test, did not consider that complement must be produced in the body and perhaps can be produced in excess.

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[36] Mayer, M. M., Osler, A. G., Bier, O. G., and Heidelberger, M., The Activating Effect of Magnesium *and Other Cations on the* Hemolytic Function of *Complement.* J. Exp. Med. 84: 535, 1946. See also Kabat, E. A. and Mayer, M. M., Experimental Immunochemistry, Springfield, Ill., 1948. Charles C. Thomas.

[37] Fischel, E. E., Pauli, R. H., and Lesh, J., Serological Studies in Rheumatic Fever, J. Clin. Invest. 28: 1172, 1949.

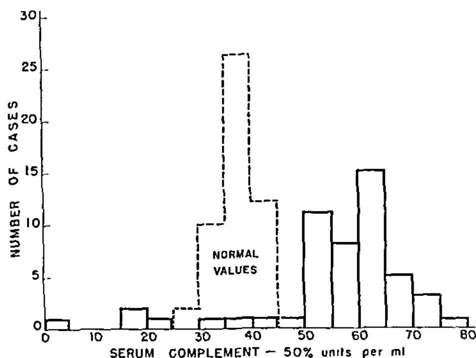


Fig 37 Serum complement values in normal adults and children compared to patients with rheumatic fever

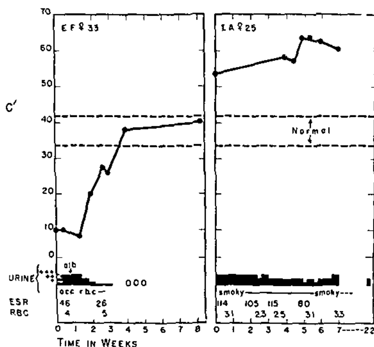


Fig 38 Serum complement pattern in acute nephritis

In rheumatic fever, there had been reports of a low complement, and we were rather surprised to find predominantly a high complement. We now have about 100 cases which we studied serially over the years. In four instances a low complement was found Two were in moribund patients and two with low C'1 for no known reason. Many non-specific things will cause a low complement, as will be discussed

We have naturally done a fishing expedition, as others have done in the past, and have found that in most inflammatory conditions, the most usual phenomenon we find is elevation of complement.

There a few very limited conditions in which we find a low complement. The one of chief interest to you probably is acute nephritis. We are not too happy about the technical difficulties in measurement of nephrotic serum, and we haven't done too much of it, but we will come to that in a moment. Lupus erythematosus has a low complement. It has been described by many people, and this, too, has particular ramifications which do not suggest an allergic reaction to us. Serum sickness has a low complement, but a few instances of the reverse have been observed. Moribund individuals with overwhelming septicemia, may occasionally present with a very low complement

There are, therefore, conditions with a low complement which may not necessarily be due to fixation of complement by antigen and antibody That which is being produced may possibly cease being produced by toxemia or death. There are also anti-complementary substances in the blood. In lupus erythematosus the gamma globulin has been well described as anti-complementary by several investigators. There are also problems of loss in the urine. We touched on that previously as well as the technical difficulties of measuring complement in various sera.

The other unexpected finding was that with a number of allergic reactions no complement fixation was observed, but rather an elevation of C'1 as in non-specific inflammations This was observed in many cases of penicillin sensitivity, Arthus reactions due to heparin, and sulfadiazine hypersensitivity of the massive overwhelming type similar to good, old-fashioned serum sickness. In periarteritis nodosa, which is less well defined as an allergy, an elevation of serum complement was found in about 20 cases Therefore, complement level alone cannot be used as an indication of an allergic reaction on the one hand, or as proof that something is not an allergic reaction on the other. In rheumatic fever, for example, we feel that we haven't confirmed the falls of C' which were reported in the literature but at the same time, we haven't disproved that it is an allergic reaction since we are not capable of doing so with complement data alone Penicillin sensitivity, for example, gives the same elevation of complement.

The next problem we were faced with was, how much of our failure to confirm previous reports in rheumatic fever was simply due to the method we employed for measuring C'. Were we dealing with a method which precludes the measurement of a low complement? Were we introducing artifacts? Did the introduction of magnesium and calcium prevent us from finding what previous investigators had reported?

This is a real problem because I think the way the old titrations were set up with double dilution methods, precluded the detection of an elevation of complement.

To get increase from the normal, by double dilution technique, for example, one would have to have 100% increase to detect a rise in serum complement. This may explain why an increase in C' was rarely if ever recognized by the old methods -- and by "old," I am also talking about the turn of the century when some very beautiful work was done by many investigators. We, therefore, decided to go back and study other diseases in which a low complement was described. In a very brilliant study, Kellett and Thomson in England in 1939 [38] demonstrated low complement in acute nephritis. Using a 50% hemolytic method, they also found a normal complement in chronic nephritis, and felt they could use C' to differentiate the two nephritides. The toxemia of pregnancy associated with nephritis was also associated with normal complement. They therefore proposed the complement test as a differential test between acute nephritis and other kinds of nephritis.

In our study with Dr. Gajdusek, who was working at Babies' Hospital, and then in Cincinnati, we accumulated some 30 cases, 18 of whom were seen within the first 10 days of symptoms of acute glomerulonephritis [39]. Of the 18, all but one had a low serum complement early in the course (Table 6). The one exception was a patient (No. 14) with a penicillin rash who had a normal complement. This points up the balance, if you will, between the consumption or inactivation of complement, if that is what is going on, and the increased production of complement due to another inflammatory stimulus, namely, a penicillin reaction This patient subsequently went on to have an elevated titer, 55 units, with subsequent return to normal. Most of the patients with nephritis had a low complement only transiently. From the quantitative aspects, however, and those are the aspects that we are particularly interested in, this suggests an overwhelming union of many antigens with many antibodies.

This, too, I think, is a point worth dwelling on. There are any number of antigens which may react with their antibodies even in the experimental nephrotoxic system, and it is not surprising that these antigens may in some instances be shared in common by liver, serum, and kidney.

We have for example, the two patients with rheumatic fever and low complement. These patients showed no evidence of renal disease, no peculiarities or differences in their mild rheumatic disease from the other 90 odd rheumatic fever patients who had high C' Some fortuitous antigen was probably uniting with some antibody, if we think of it in those terms.

There is no parallel between the low C' and the urinary abnormalities of acute nephritis. Again, this is to be expected because it is likely that those antigens and antibodies which are responsible for pathogenicity may continue to exert a profound effect on a small area of the kidney, with quantitatively little effect on total body complement. On the other hand, such antigens and antibodies may have long since ceased their troublesome work while more benign antigens and antibodies continue to cause fixation of complement. One patient (No. 22) went on with a zero level of

[38] Kellett, C. E. and Thomson, J. G., Complementary Activity of Blood Serum in Nephritis. J. Path. and Bact. 48: 519, 1939.

[39] Fischel, E. E. and Gajdusek, D. C., Serum Complement in Acute Glomerulonephritis. Am. J. Med. 12: 190, 1952.

TABLE 6

SERUM COMPLEMENT (C') IN ACUTE GLOMERULONEPHRITIS*

Case No.	Days after Onset									Remarks
	2–4	5–7	8–10	11–14	–21	–28	–35	–42	–49	
1	18	46(a)		35						(a) Periapical abscess end of 1st wk
2(b)	11	10		9 14	20	27,26	38	40		(b) See Fig 1, I F
3	10	10								
4	10	18	12		11					
5	17	32		43						
6	17	47		36	30	28				
7		21,21			39					
8		29				48				
9		30	43		57					
10		15								
11		16	18 18		20					
12		0	12	20						
13		19		30						
14		33(c)	55	38						(c) Penicillin rash
15			19		40,19		31		33	
16			26							
17			26		0(d)	31	35	34		(d) Clinically worse 42 in 10th wk
18			28(e)			23		26		(e) Left otitis and hemolytic streptococcal pharyngitis
19				34	39					
20				37	40	49 54	50		51	32 in 11th wk
21				54(f)	50	73 62	65		61	(f) Concurrent rheumatic fever
22					0,0			0		0 in 8th wk 0 in 12th wk, 40 after 1 yr
23					30 31	44				
24					38	38				
25					39	34				
26					36	37	39			
27					14			24		
28					14,24		63(g)	81	65	(g) Developed thyrotoxicosis
29							38	47	41	39 in 8th wk
30							30	41		

* 50% hemolytic units of C' per ml serum in relation to duration of disease

complement for at least 12 weeks although the hematuria, albuminuria and other manifestations of disease cleared relatively early, before the seventh week. The fact that at the one year follow-up his complement titer was found to be normal was satisfying

Despite the low complement, the body is able to produce large amounts of complement when a stimulus which we usually associate with complement production is present. A patient (No 1) with an initial level of 18 units developed a level of 46 units with a periapical tooth abscess Other causes of high complement in this series were penicillin rash, (Case 14) concurrent rheumatic fever, (Case 21) and the development of thyrotoxicosis (Case 28) while on the wards The simple statistical data do not mirror the constant anabolism and catabolism of complement in the body, particularly when other conditions complicate the picture. The amounts of antigen and antibody required to fix complement in vivo from the total blood volume in order to obtain a very low complement titer are also of interest, as Dr. Stavitsky pointed out. We began a study of this with Dr. Benacerraf and have recently renewed this study with Dr. Nordén Guinea pigs passively sensitized with a minute amount of anti-egg albumin can be killed of anaphylactic shock without any deviation of complement. When given a large amount of antibody, however, a deviation of complement will occur, as might

be expected from quantitative studies on complement fixation in the test tube. This data has not been published as yet. We may not have controlled all the variables such as repeated bleeding in such small animals. A few animals given the antigen alone and bled from the heart for baseline complement titer occasionally have a striking increase in titer on the following day. Exposure to trauma and an innocuous antigen, egg albumic, results in a rise in C'. Complement was once defined in a negative way as something that did not increase when the antigen was injected, in contradistinction to specific antibody. Yet here, with precise techniques, or other conditions a different picture is obtained. At any rate, the quantitative fixation of complement in vivo probably follows the pattern observed in vitro, but is more difficult to control.

Injecting normal rabbit serum into our guinea pigs will sometimes cause an anti-complementary effect. This also complicates the evaluation of fixation produced by specific allergic reactions. If we are going to translate these data to clinical interpretation in nephritis or nephrosis, there is much that has to be qualified. Table 7 uses what we considered at the time a feasible term -- subacute glomerulonephritis. We didn't like the term "subacute", but we used it merely in those cases which by virtue of their duration could not be categorized with certainty as either acute nephritis subsiding, or well established chronic nephritis. The latter diagnosis carries with it a much more ominous prognosis, and calls for some hesitation before making it. In these patients with "subacute" and chronic nephritis we found a variable picture. In some during 2-9 weeks of complement determinations, there was a suggestion of a low value; in others there was a high value. Case 31 developed a nephrotic syndrome, and we had low complement titers. Case 38, on the other hand, did not develop a nephrotic syndrome and values were 5, 5, 5, 0, 0, 5 between the fourth and sixth month. Cases 39, 40 and 41 had uremia, two of them dying within a short period of time. All had levels of complement above normal.

TABLE 7

SERUM COMPLEMENT (C') IN "SUBACUTE"* AND CHRONIC GLOMERULONEPHRITIS†

Case No.	Time after Onset during Which C' Was Determined	Serum Complement Values— 50% Hemolytic Units	Remarks	Months' Duration of Definitely Abnormal Urinary Findings at Last Examination
31	2 to 9 wk	46 38,29 27,23	Developed nephrotic syndrome in 4th wk., chronic glomerulonephritis	18
32	3 to 4 wk	14,15,17,26	"Subacute" glomerulonephritis	8
33	1 to 7 mo	53,58,57,63,63,62 60 53	Chronic glomerulonephritis (Fig 1 I A.)	10
34	4 to 8 wk	59,53,52,50 53	"Subacute" glomerulonephritis	5
35	7th wk	43	Chronic glomerulonephritis	28
36	3 to 4 mo	47,13 15,21,15	Developed nephrotic syndrome chronic glomerulonephritis	14
37	4th mo.	46	"Subacute" glomerulonephritis	4
38	4 to 6 mo	5 5,5,0,0 5	"Subacute" glomerulonephritis	10
39	1 yr known nephritis	82,69	Uremia, died 1 mo later	24
40	14 yr known nephritis	52	Uremia and heart failure temporarily recovered	14 yr
41	2 yr known hypertension	55	Uremia, died 3 days later	

* "Subacute" is used for cases which by virtue of duration cannot be categorized with certainty as either acute or chronic glomerulonephritis.

† 50% hemolytic units of C' per ml serum in relation to the duration of disease

Figure 38 is a pattern of serial determination of complement in one of our patients (E.F.) with acute nephritis. In this instance there appears to be a coincidence between the clearing of urinary findings and the rise to normal of complement. The second chart of Figure 36 is of a patient (I.A.) who on admission was thought also to have an acute nephritis. In follow-up, however, it appeared that she had chronic nephritis.

At 22 weeks after admission, she still had *two plus albuminuria. In this case,* as Kellett and Thomson originally described in chronic nephritis, there is no reduction in complement. They described a normal C' but we find an increase which serves to emphasize the difference from uncomplicated acute nephritis. Table 8 shows the C' in other conditions associated with proteinuria and this is another argument against the suggestion that C' loss in the urine accounts for the low C' in acute nephritis. In the first example given, a patient with left lower lobe pneumonia with a 4 plus proteinuria, the complement was normal on the second day and went up to 63 by the seventh day. Carbon tetrachloride poisoning and exogenous poisons which couldn't be defined, are associated with striking elevation of complement up to 70, 80 and in one case 90 units. In Case 48 with so-called ischemic or lower nephron nephrosis the complement was found low when the patient was moribund, and subsequently it became elevated. Three patients with so-called lipoid nephrosis showed variation -- two with low figures, not strikingly low -- 21, 25 and one elevated at 62. One patient with Kimmelstiel-Wilson syndrome had a normal complement at the time we studied the serum.

TABLE 8

SERUM COMPLEMENT (C') IN OTHER NEPHROPATHIES AND CONDITIONS SIMULATING ACUTE GLOMERULONEPHRITIS

Case No.	Day of Illness	C' 50% Units per ml Serum	Diagnosis	Urine Findings
42	2	44	Left lower lobe pneumonia	Albumin 4 plus, RBC loaded, few WBC, cleared after 1 wk
	7	63		
43	14	60	Pyelonephritis due to B coli and Staph aureus hemolyticus	Albumin 3 plus, 40–60 RBC, few WBC
44	10	58	Nephritis and mild hepatitis due to carbon tetrachloride	Albumin 3 plus, many RBC, 2–4 WBC, many casts, clearing in about 1 wk
45	9	57	Nephritis due to ? carbon tetrachloride *elevated blood* urea nitrogen	Albumin 4 plus, rare RBC and WBC, clearing gradually after 3 wk
	21	90		
	28	72		
46	22	80	Lower nephron nephrosis due to undetermined exogenous poison	Albumin 4 plus, clearing gradually over 6 wk
47	11	51	Nephrosis due to bacitracin	Albumin 4 plus, gradually clearing in 2 wk
48	? 5	29(a)	Lower nephron nephrosis, shock and transient uremia following induced abortion no infection	Albumin 2 plus, 20–30 RBC, occasional WBC
	22	66		
	26	56		
	33	53	(a) = almost moribund	
	40	53		
49	45	21	'Lipoid' nephrosis	Albumin 4 plus, occasional WBC
50	±1 yr	62	Lipoid" nephrosis	Albumin 4 plus, occasional WBC
51	10	25	"Lipoid' nephrosis	Albumin 4 plus, occasional RBC and WBC, no change
	17	39		
	24	29		
	31	22		
52	58	46	Diabetes mellitus nephrosis, (Kimmelstiel Wilson syndrome)	Albumin 3 plus microscopic, negative

Finally, a word about complement as indication of allergy. In acute glomerulonephritis, the low C'level is one additional factor which added to all the others is suggestive of allergy. The relationship to hemolytic streptococcal infection, the latent period, the relatively abrupt and acute onset of a disease which usually has a good prognosis, all speak for a sudden allergic reaction, *if you will, and the low complement* is compatible with that finding.

When it comes to nephrosis, I am afraid that I can't go along with the idea that this proves an allergic basis for the disease. There are no other clinical data as there

are with acute nephritis. If the supposition that nephrosis is due to antigen-antibody interreaction rests solely on the low C' levels found, then attention should be called to the many other factors which result in a low C' and to the technical difficulty of measuring C' in nephrotic serum.

I haven't tackled it myself. I hope Dr. Ruth Alice Davis and Dr. Riley will mention some of their work in which they have tried to separate out the lipemic substances and then determine complement. It is a very difficult thing to do. Perhaps, if you could separate off the fat layer the serum C' would not be too low in terms of water content of the serum.

Other problems include, as has been mentioned, possible anti-complementary action of lipids. It might be anticipated from the low serum protein, as was suggested, that complement along with the other proteins might be deficient in nephrotic serum. To speak of a low C' as a proof of pathogenetic mechanism in this instance is, I think, a little premature at this time.

Mention was made of ACTH and Cortisone this morning. Again we do not feel that change in complement titer during hormone therapy necessarily mirrors an allergic reaction. I think we have been partly guilty along with others of demonstrating an inhibition of antibody production with massive doses of Cortisone and ACTH [40]. The inhibition of antibody production, although marked, is relatively slight in terms of the amounts needed to alter passive hypersensitivity reactions.

We produced quantitative, passive Arthus reactions with a known amount of antibody and antigen, single antibody, single antigen presumably, and found that the administration of rather large doses of Cortisone did not inhibit these reactions [41]. The changes in C' observed in hormone treated nephrotics are a little too fast to attribute them to diminished allergy secondary to altered antibody production. Quantitatively, the changes are probably not significant enough to interrupt any allergic reaction if such exists.

The change in C' may perhaps be better thought of as paralleling changes in proteins generally. The hormones seem to make all deviations from the normal revert toward normal. Whatever the reason, I don't know, but it is of interest that Dr. John Vaughan, who is now in Richmond, reported from Boston that in patients with lupus given ACTH and Cortisone, a rise in complement occurred [42]. Of course, he found

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[40] Bjorneboe, M., Fischel, E. E. and Stoerk, H. C., The Effect of Cortisone and Adrenocorticotrophic Hormone on the Concentration of Circulating Antibody. J. Exp. Med. 93: 37, 1951.

[41] Fischel, E. E , Adrenal Hormones and the Development of Antibody and Hypersensitivity, in Effect of ACTH and Cortisone upon Infection and Resistance G. Shwartzman, Editor, Columbia University Press, 1953.

[42] Vaughan, J. H., Bayles, T. B. and Favour, C.B., Effect of 17-Hydroxy-11-Dehydrocorticosterone and Adrenocorticotropic Hormone Upon Plasma Gamma Globulin, Fibrogen, and Erythrocyte Sedimentation Rate. Proc. Soc. Exp. Biol. & Med. 76: 274, 1951.

a low C' to begin with. In our patients with any non-specific inflammatory disease who initially have a high C' and to whom we give Cortisone, the converse occurs. As you would expect, during hormone administration all aberrant levels seem to revert toward normal.

One other observation: in patients with schizophrenia being given large doses of ACTH, we had the opportunity to test for complement and found no appreciable changes during rather severe hyperadrenal states over a long period of time. In the medically normal individual, if you will, I don't think we can affect complement too much with hormone Naturally, in those who developed complications due to hormone therapy, we had deviation. Thank you.

CHAIRMAN RAPOPORT. Dr. Riley will assume the chair for the next part of the session

III. EXPERIMENTAL RENAL VASCULAR DISEASE

A. RENAL LESION OF THE SCHWARTZMAN REACTION

CHAIRMAN CONRAD RILEY: Dr. Thomas, will you talk about Renal Lesion of the Schwartzman Reaction?

DR. LEWIS THOMAS: I am planning to talk to you briefly about an experimental form of diffuse vascular disease which resembles, in certain of its morphological aspects other disease states that have for many years been regarded as allergic in basis, although the mechanisms which appear to operate in the production of these lesions do not involve a recognizable disturbance of a known immune system, it is possible that a process analogous to bacterial hypersensitivity may be implicated.

The endotoxins of gram negative microorganisms are perhaps the most ubiquitous and most widely distributed bacterial toxins in nature. They are demonstrable in all gram negative organisms that thus far have been studied for their presence. They are not possessed, on the other hand, by any known gram positive organisms. Chemically, in so far as we can cateogorize them, they comprise a fairly homogenous class of substances. They appear to be macromolecular complexes, consisting of lipid, protein and polysaccharide fractions, and an as yet unidentified phosphorous-containing component. When injected into experimental animals, they produce a stereotyped series of physiological reactions, and the variety of these reactions has given rise to some confusion in the nomenclature of the endotoxins. Depending upon what your field of interest is, you may recognize them by one or another descriptive term.

For example, these are the "bacterial pyrogens". It is most likely that the substances in contaminated blood transfusions or infusions fluids which give rise to fever and shock, and the active components of the so-called "foreign protein" therapeutic agents, are in all instances the endotoxins of one or another of the gram negative microorganisms

The endotoxins are also the substances which, when injected by vein, produce hemorrhagic necrosis in rapidly growing animal tumors. They are, therefore, known to some as "tumor-necrotizing toxins."

The endotoxins also have the capacity of producing a considerable degree of hyperglycemia within an hour or two after injection, followed sometimes by lethal levels of hypoglycemia. They are also the materials which produce the Shwartzman reaction, and therefore they have become known to some as the "Shwartzman-active toxins."

The local Shwartzman reaction is produced by an intradermal injection of endotoxin, followed 24 hours later by an intravenous injection of the same material. The site of the intradermal injection, which undergoes a brisk inflammatory reaction during the stage of "preparation", becomes involved in an extensive reaction of hemorrhagic necrosis within two hours after the intravenous injection. A modification of this phenomenon, known as the generalized Shwartzman reaction, is the problem which concerns us this afternoon

The generalized Shwartzman reaction is produced in the following fashion: Rabbits, preferably young rabbits, are given a preparing injection of endotoxin by the intravenous route, instead of into the skin. They are then allowed to rest for 24 hours, and at the end of this time another intravenous injection is given Within 2 or 3 hours after the second or "challenging" injection of endotoxin, the glomerular capillaries become filled with a homogenous eosinophilic material which completely jams the lumen, and this occlusion of the glomerular circulation is followed within the next few hours by the development of bilateral cortical necrosis of the kidneys.

One of the central mysteries of the reaction is the role--the evidently dominant role--which is played by time in its pathogenesis One can give any amount of endotoxin in a single intravenous injection, up to lethal doses, without the development of bilateral cortical necrosis of the kidneys. But when one gives two injections, spaced 18 to 24 hours apart, amounts of endotoxin which are far below the lethal dose produce bilateral renal cortical necrosis in as high as 100% of the animals

Not only must there be a time interval, but it must be an interval of the proper duration. For example, if the interval between the two injections is shortened to 2, 4, or 8 hours, the animals do not develop kidney lesions. The optimal interval is somewhere between 18 and 24 hours

It is evident, then, that during a period of time after the first injection of endotoxin, some change occurs which renders the animal excessively susceptible to a new effect of endotoxin There is a paradox in this. Most of the untoward reactions which are displayed by rabbits following one injection of endotoxin occur during the first few hours after the injection is made. This is the time during which the animals develop high fever, or go into shock, or exhibit hyperglycemia or hypoglycemia This is also the time during which severe polymorphonuclear leucopenia occurs But on the following day, when they have become fully prepared for the generalized reaction, they seem to be in reasonably good health on all counts.

It is our purpose this afternoon to present evidence which suggests that there are two main participants in the generalized Shwartzman reaction One of these appears to be fibrinogen, or an altered form of fibrinogen. The other seems to be a material which has the property of causing precipitation of fibrinogen within the lumen of small blood vessels Before considering the evidence in support of this view, let us review briefly the histological changes which characterize the generalized Shwartzman reaction.

During the first two hours or so after the second injection of endotoxin, the glomerular capillaries become occupied by a homogenous, sometimes slightly granular,

acellular, eosinophilic substance, which is at first laid down in layers along the inner surface of the capillary wall and subsequently fills the entire lumen as a solid mass. Occasionally, there may be tiny strands of fibrillar material in the capillaries, but in general the deposit does not resemble fibrin It has more the appearance of "fibrinoid." Furthermore, it presents all of the staining reactions which are considered to be characteristic for fibrinoid, according to the criteria of Angevine and Altschuler, including an intense purple stain in the Hotchkiss-McManus modification of the Schiff reaction.

In fresh preparations prepared from homogenates of renal cortex, the glomeruli containing deposits of fibrinoid are quite resilient, almost rubbery objects. Homogenates can be prepared by prolonged treatment in a Potter homogenizer or a Waring blender, and the glomeruli retain their shape quite nicely. When viewed with ordinary light, saline suspensions of the unstained glomeruli show the presence of a colorless or sometimes faintly yellow material inside the capillaries. But when the glomeruli are viewed in polarized light, the intracapillary material is seen to be extremely birefringent. The birefringence is not effected by exposure of the glomeruli to lipid solvents. It withstands elevations of pH to 10 or higher without being altered. It disappears, however, within a half hour or so after the pH has been reduced to 2.5 or 3 Significantly, the birefringence disappears within a few minutes when the glomeruli are exposed to trypsin or papain. These observations suggest that the material contained in the capillaries is a protein of highly organized molecular structure.

The same sort of pink staining fibrinoid material appears in many other intravascular locations in animals with the generalized Shwartzman reaction. It is present in the sinusoids of the liver and spleen, and is deposited in extensive masses beneath the intima of the walls of the coronary arteries. It is also demonstrable as acellular vegetations on the mitral and aortic valves of the heart.

The appearance of fibrinoid material within the small blood vessels, and the gross hemorrhagic and necrotizing lesions of the generalized Shwartzman reaction, can be prevented by the administration of heparin at the time of the second injection of endotoxin. The animals must be given a sufficient amount of heparin so that the blood is incoagulable for three or four hours after the second injection; in this circumstance it is possible to prevent the reaction in all animals.

In the course of a study of the mechanism involved in this effect of heparin, we have observed a new reaction in the plasma of animals, which occurs after an injection of endotoxin, consisting of the precipitation by heparin of a large volume of protein material at low temperatures. The easiest method for demonstration of the material is to obtain heparinized blood in the ordinary fashion, remove the cells by centrifugation, and place the plasma at 4° C. for a period of 30 to 60 minutes. During this time, in animals which have received endotoxin during the preceding 1 to 4 hours, an opaque flocculant precipitate begins to form within about 15 minutes after chilling the tubes. It increases in density and amount and eventually settles to the bottom of the tube as a solid mass. When estimated in terms of dry weight, or by the biuret reaction, the material constitutes between 50 and 150 mg. per cent of the plasma. There are reasons to believe that the material precipitated by heparin is closely related to fibrinogen. In the first place, the washed precipitate has been shown to be clottable

by the addition of thrombin. Secondly, plasma from which the material has been removed by centrifugation has been found to contain greatly diminished levels of fibrinogen. Thirdly, the washed precipitate, redissolved in warm saline or buffer, has been found to migrate as a single component in paper electrophoresis, and its migration is similar to that of fibrinogen. Finally, we have found that heparin-precipitation only occurs in plasma and never in serum, indicating more strongly the likelihood that it is related either to fibrinogen or to fibrin.

The precipitable material does not appear in the plasma of heparinized rabbits, when heparin is administered prior to the injection of endotoxin It seems possible that the material may be an altered form of fibrinogen, perhaps polymerized fibrinogen, representing a form of this material somewhere midway in the transformation of fibrinogen to fibrin

The observation suggested that a particular affinity existed between heparin and the altered fibrinogen It was therefore of interest to learn that Walton, in the course of studies on the properties of synthetic preparations of dextran sulfates, had observed that dextran sulfates of large molecular size possessed anticoagulant properties similar to those of heparin, and also were capable of combining with and precipitating fibrinogen when added in optimal concentrations. Moreover, Walton reported that the intravenous injection of large amounts of dextran sulfate resulted in the development of glomerular lesions which were strikingly similar to those observed by us in the generalized Shwartzman reaction. The glomerular capillaries were filled with homogeneous, eosinophilic material with staining properties similar to those of fibrinoid. Hausman and Dreyfus reported similar observations with another acidic polymer of large molecular size, sodium polyanethol sulfonate, known to the trade as Liquoid. This material is an anticoagulant, resembling heparin, and also is capable of causing precipitation of fibrinogen from plasma. When injected into animals in sufficiently high doses, it has been reported to cause occlusion of the glomerular capillaries by material resembling fibrinoid

We have found that an injection of gram negative bacterial endotoxin enormously enhances the toxicity of these acidic polymers, and makes it possible to produce the generalized Shwartzman reaction consistently within a very short period of time, using doses of dextran sulfate or Liquoid which are of themselves completely nontoxic for animals The amounts of endotoxin which are required for this synergistic effect are extremely small. For example, we have produced bilateral cortical necrosis of the kidneys using 2 micrograms per rabbit of purified dysentery endotoxin, or with dilutions of meningococcal endotoxin as high as 1 to 10,000. The optimal time for the injection of acidic polymers has been found to be between 1 and 2 hours after the injection of the endotoxin. It is of particular interest that dextran sulfate or Liquoid are most effective in producing renal lesions when they are injected at the time when the highest concentrations of heparin-precipitable fibrinogen exist in the plasma. We were led by this observation to the tentative hypothesis that fibrinogen is in some way involved in the formation of fibrinoid. In order to test this theory, the levels of fibrinogen before and after an injection of acidic polymer were determined in normal rabbits, and in rabbits which received an injection of endotoxin an hour or so prior to the administration of acidic polymer. The results were striking. In every instance, it was found that the level of fibrinogen was not significantly altered during a period

of one hour after the injection of Liquoid or dextran sulfate, in normal rabbits. However, in *animals previously injected with meningococcal endotoxin, the injection* of acidic polymer was followed within ten minutes by the disappearance from the blood of all, or almost all of the plasma fibrinogen. To illustrate, a group of nine rabbits were injected with meningococcal endotoxin, and this was followed 2 hours later by an intravenous injection of Liquoid. The animals were then bled ten minutes after the injection of Liquoid, and again at the end of one hour. The average fibrinogen levels for the group prior to the injection of Liquoid were in the vicinity of 300 mg.%. Ten minutes after the injection of Liquoid, the fibrinogen had fallen to less than 50 mg.% and remained at this low level for an hour. After two or three hours, in animals surviving the experiment, fibrinogen began to reappear in the blood.

Thus, we have observed that during the most crucial period in the development of bilateral renal cortical necrosis, when deposition of fibrinoid material is occurring within the capillaries, the fibrinogen of the blood becomes greatly depleted. In animals who are protected against the generalized Shwartzman reaction by the administration of heparin, no fall in the level of fibrinogen occurs. On the basis of this circumstantial evidence we suggest that the origin of fibrinoid, in this particular experimental circumstance, may be fibrinogen.

Several years ago we observed that the generalized Shwartzman reaction could be prevented by nitrogen mustard, and the protective effect of mustard seemed to be related to its capacity to produce polymorphonculear leucopenia. It was found that the Shwartzman reaction was prevented only during the relatively brief period of time during which the neutrophiles disappeared from the blood, three days or so after the administration of nitrogen mustard. Moreover, if the animals were given nitrogen mustard and at the same time prevented from developing leucopenia, by clamping the femoral artery for a period of five minutes during the administration of nitrogen mustard, leucopenia did not occur, and such animals were not protected against the generalized Shwartzman reaction We concluded at that time that the circulating polymorphonculear leucocytes were in some way concerned in the pathogenesis of the reaction.

It therefore became of interest to determine whether nitrogen mustard prevented the effect of the synthetic heparin-like polymers. We found that this was not the case. Despite the production of absolute neutropenia by nitrogen mustard, the administration of endotoxin combined with Liquoid or dextran sulfate produced the same incidence of bilateral cortical necrosis as in control animals not treated with mustard. The observation suggests that the acidic polymers may be capable of substituting for, or imitating, the role which is played by leucocytes in the conventionally-produced generalized Shwartzman reaction. Conceivably, the role of the leucocytes may be to contribute some substance which has properties similar to those of acidic polymers, and which can therefore combine with and precipitate fibrinogen in the circulating blood, leading to the deposition of fibrinoid.

In summary, we would suggest that two participants in the generalized Shwartzman reaction which lead to the appearance of intravascular fibrinoid are fibrinogen, altered in some way so that it can combine with and be precipitated at low temperatures by heparin, and an unknown polymerized material of large molecular size, with

combining properties similar to those of heparin, which causes precipitation of fibrinogen within the circulating blood.

The alteration in fibrinogen which leads to its precipitability by heparin has been found to occur to an equal extent after either of the two intravenous injections of endotoxin, but it persists for only a few hours. Perhaps the role of timing in the pathogenesis of the generalized Shwartzman reaction is to allow the two participants to become present at the same time. The function of the preparing injection may be to produce an effect on the leucocytes which results in the availability, 24 hours later, of a polymerized substance with the properties of dextran sulfate or Liquoid. By itself, this event would not lead to damage But a second injection of endotoxin, given at this time, causes the appearance of fibrinogen in its precipitable form, and the final result is the deposition of fibrinoid material within the glomerular capillaries.

Obviously this is hypothesis, based on studies which are still in progress. It has the advantage, we believe, of offering a new approach to the problem of fibrinoid which can be directly studied by experimental manipulation.

DR. DAVID GITLIN: We just concluded a study of the fibrinoid -- so-called fibrinoid degeneration that takes place in humans. We have been unsuccessful thus far in producing the Shwartzman Reaction in young rabbits, whether it be with Shear's polysaccharide or endotoxin. In humans, we have seen two patients, two children, who prior to death appeared to have what might be considered a generalized Shwartzman Reaction, and on post mortem examination they demonstrated lesions which are identical with those you have demonstrated today

DR THOMAS: Had they received cortisone?

DR. GITLIN: Only immediately prior to death in one and not in the other. In both of these instances, we had an opportunity to stain sections by a method that was previously described before this group, using an antibody which is specific to fibrin, using Coombs fluorescent antibody procedure, and there is very little question in our minds that this material which we call fibrinoid is some derivative of fibrinogen, presumably fibrin. Just to go a bit further, the fibrinoid degeneration we see in rheumatoid arthritis, rheumatic fever, and many other conditions, is apparently fibrin and not degeneration of collagen at all.

DR. OSCAR D. RATNOFF. I want to ask Dr Thomas a number of questions which he may or may not have the answer to.

In clinical states, there are a number of conditions in which the reaction between fibrinogen and thrombin appears to be altered. Now, in some cases this may be due to changes in constituents of the plasma other than the fibrinogen, but in a number of instances which are intriguing to me, the fibrinogen itself appears to be altered.

The most common of these is the newly born infant in whom the reaction between fibrinogen and thrombin is delayed, apparently, because of a change in fibrinogen itself. I wondered whether he had the opportunity to study this phase. Is the fibrinogen altered in such a manner that its reaction with thrombin had been altered?

Second, I wondered whether he had observed the appearance of the heparin precipitable protein in clinical states because there is one clinical state which might have been misinterpreted, as you look at the data, and that is premature separation of the placenta. It may become defibrinated, I always thought, because of thromboplastic activity of the placental tissue, but the subsequent course of these ladies at times is such that they go on to develop a renal lesion not unlike the cortical necrosis of the generalized Shwartzman's phenomenon.

Finally, many substances have one obvious in vitro effect, and then a few more subtle effects. I think that the obvious example of this is tissue thromboplastin which everybody knows is thromboplastic, but as has been shown by Hartman and Connolly, the thromboplastic preparations are pari-pasu, antithrombic, or at least they do something to the fibrinogen-thrombin reaction.

DR. THOMAS: I would like to answer the third point first.

The most extensive studies that have been made with the acidic polymers which we used were made by Walton and Ricketts with dextran sulfate. We have repeated their observations, and confirmed them also with sodium polyanethol sulfonate. The most conspicuous in-vitro effect to appear to the naked eye is precipitation of fibrinogen.

It is almost an immunological type of precipitation. With excessive amounts of dextran sulfate, precipitation fails to occur, and a precipitate formed with optimal concentrations redissolves when more dextran sulfate is added. The presence of heparin prevented the precipitation of fibrinogen by dextran sulfate.

As far as the second point is concerned, we have engaged on a fishing expedition, to borrow a phrase, with patients with a variety of different diseases, and the highest levels of heparin-precipitable fibrinogen we have encountered yet have been in children with acute rheumatic fever and rheumatoid arthritis. It also appears to be elevated in pregnancy, and in the acute stage of various infectious diseases.

In patients with rheumatic fever in whom large masses of precipitable material are present, the administration of cortisone is followed within a few days by disappearance of the material, paralleling the expected fall in the sedimentation rate. Whether this kind of fibrinogen has to do with sedimentation rate, we do not yet know.

DR. HEYMANN: Is the renal lesion associated with hyperlipemia?

DR. THOMAS: Hyperlipemia occurs in many of these rabbits as the result of preparation for the generalized Shwartzman Reaction, and it exists at the end of twenty-four hours, before the second injection of endotoxin is given.

The cholesterol levels reach three or four times the level of normal as the result of one injection of endotoxin.

DR. FISCHEL: One of the peculiar things about this system is the fact that animals will apparently adapt to it so that repeated induction of Shwartzman reaction

in the skin, at any rate, becomes less productive of a lesion. Bennett and others have studied this in relation to colloidal material. I wonder if you have studied this in relation to this system and found correlation.

DR. THOMAS: Yes, we have. We have found that induced resistance to the generalized Shwartzman reaction appears after a series of daily injections of sublethal amounts of endotoxin. In such animals, heparin-precipitable material does not occur in the plasma following an injection of endotoxin.

DR RAPOPORT: Have you examined the kidneys of patients in different stages of the nephrotic syndrome? Dr. Erich has stated that patients with the nephrotic syndrome who develop progressive glomerular disease, show as the first sign of this progress, a layering of fibrinoid material in the capillary walls.

DR. NORMAN KRETCHMER. Dr. Thomas, what happened to the tubules during this generalized Shwartzman Reaction?

DR. THOMAS: During the first four hours nothing histologically demonstrable happens, and then they participate in the general hemorrhagic necrosis which involves the entire cortex.

There is one change of interest, though. That is, that during even the first two or three hours, material which morphologically is similar to the intracapillary fibrinoid material appears in some of the tubules, and it is our impression some of it must be getting through and being excreted very early

DR KRETCHMER: Is it only in the proximal tubule that you see this material?

DR. THOMAS: Early only in the proximal tubule, but later on with animals with full-blown reaction, we have seen it down in the collecting tubules.

DR. JAMES BAXTER: I didn't understand about heparin and other polymers.

DR THOMAS: Heparin and dextran sulfate are possibly competitive Heparin may protect by combining with fibrinogen before dextran sulfate, forming a complex which remains soluble at body temperature.

B. EFFECTS OF TEMPORARY INTERRUPTION OF RENAL BLOOD FLOW IN RATS

CHAIRMAN RILEY: Dr. Koletsky, now will tell us about the Effects of Temporary Interruption of Renal Blood Flow in Rats.

DR. S. KOLETSKY: What I have to say is in the line of exposition. I don't propose to prove anything.

We got interested in the effects of shutting off blood flow to different organs during the war. Some of you may be familiar with the technique of wrapping a rubber band around the upper thigh of an animal, keeping it on for four or five hours, and after it is removed, there is a massive loss of fluid into the injured leg, and the animal goes into shock.

We spent a great deal of time looking for a so-called toxic substance that was supposed to come from the area of injury, circulate and produce shock. We never found that substance, but in the course of thinking about the effects of shutting blood flow off, we thought we would try this on the kidney, particularly since a good deal of emphasis was being placed by clinicians on a syndrome that they called the shock kidney.

This was a kidney which was supposed to be severely injured because it was deprived of its blood flow in states of shock through low blood pressure and vasoconstriction Subsequently, after the shock was successfully treated and the patient seemed to recover, progressive anuria or total anuria followed and the patient died in uremia. This was a very puzzling situation. It was attributed to the effects of very small and almost negligible blood flow in the kidney itself.

Several pathologists were interested in this condition. They studied the lesion very thoroughly, and evolved the concept of lower nephron nephrosis.

This was a very peculiar thing in itself because here was an injury that was supposed to be very specifically localized to one given segment of the nephron, the distal part of the convoluted tubule, including the loops of Henle. I think a moment's reflection suggests that if you render the kidney totally anoxic or markedly ischemic for a period of time would produce a specific lesion in one particular segment of the nephron, just doesn't sound right. In fact, it just sounds wrong, but nevertheless, the

pathologists could only report what they saw. They said that individuals dying of this so-called shock kidney had neither glomerular lesions, nor lesions of the proximal convoluted tubules, but a specific nephrosis of the distal convoluted tubule, and this caused uremia by upsetting the capacity of the tubule to maintain what is called its normally selective capacity to reabsorb fluid, and presumably electrolytes or other substances

We thought that we could simulate the conditions of the shock kidney by interrupting blood flow to the kidneys and determining what kind of lesion there was, and what the consequences were

We had never been able to produce anything like the shock kidney as a result of shock itself This was the general experience of practically all people who worked in this field.

It is very simple to shut off blood flow to the rat kidney We merely dissected both kidneys free, isolated the pedicles and threw a long clamp across the entire hilum. This catches the ureter, the renal artery and renal vein.

No blood entered or left these organs for the period of the experiment. If both kidneys are simultaneously deprived of all flow of blood for 30 minutes, we found a little rise in blood urea nitrogen levels, ranging from say 40 to 80. Occasionally a few went a little higher. After a few days, they all recovered, and apparently lived happily after that with no apparent consequence.

Next, if one shuts off flow to both kidneys simultaneously for an hour, the blood urea nitrogen levels go up to higher levels, but most of the animals, nevertheless, seem to recover after about 5 or 6 days, but occasionally one died in uremia

If the same process is repeated for an hour and a half only a relatively small number of the animals survive, and most go on and die in uremia at about 6 or 7 days

Now, if the blood flow to both kidneys is simultaneously occluded for two whole hours, no rat will survive They develop a progressive rise in blood urea nitrogen and die in uremia somewhere around the third or fifth day

What accounted for the uremia? We found that the basic lesion was in the proximal convoluted tubule, where there was a massive type of coagulation nephrosis, a necrotizing nephrosis, not too dissimilar objectively from the type of lesion one sees in mercury poisoning.

It was very obvious even in the animals that were to die that the kidneys had apparently a remarkable tendency to heal this necrotizing lesion, again in much the same way as mercurial nephrosis, and of course, it seemed the reason many of these rats were dying was they developed complete blockage and complete tubular destruction and died before they had a chance to recover

The next thing we did was investigate this phenomenon on a unilateral basis.

Here we had one untouched kidney which would guarantee the survival of the rat, regardless of what we did. We could have clamped one kidney for 24 hours, and the rat wouldn't have died because the other kidney had been left intact.

Now, Figure 39 is a composite picture that shows you what happens to a kidney when you completely block off its blood flow for a period of two hours.

At the end of an hour and at 6 and 12 hours, the kidneys are usually a little enlarged and swollen. They show marked hyperemia of the medulla.

This seems to indicate that perhaps the Trueta shunt theory might be operative here, but during this period of return of blood flow, they are developing massive necrosis of the tubules, and they go on at the end of 5 days, and one week to a large, pale, swollen kidney of the nephrotic type. In other words, they get marked necrosis.

There is a tendency microscopically for complete tubular repair, and then a surprising thing occurs

After healing the kidney shrinks down in size so that at 2 weeks it is much smaller than the opposite one On the left you see the opposite, intact, untouched kidney. Very often at the end of 3 weeks, you have what seems to be a dwarfed kidney.

We have turned off blood flow for periods of 3 and 4 hours with the same result. There is a remarkable capacity to repair the necrosis of the tubules, but we wind up with a tiny kidney

Sometimes the kidneys were only one-fifth the size of the opposite kidney. Nothing seems to be wrong with them. Their architecture is preserved. You can still see the structure completely except that all of the nephrons are completely atrophic and collapsed.

DR. BARNETT: Is the other kidney larger by this time?

DR KOLETSKY: There is some compensatory hypertrophy. We have followed experiments of this kind out of curiosity more than anything else for long periods of time, and if you leave the opposite kidney alone, nothing will happen to this dwarfed kidney. It remains permanently small; it is not likely that it functions.

We have followed such animals for periods of 18 months to 2 years Interestingly enough, we have not observed the development of hypertension during the course of the necrotizing lesion and subsequent shrinkage and atrophy of this kidney.

I call your attention to this because I think it is significant in connection with some observations to be made later

Figure 40 shows the basic lesion. When you interrupt blood flow to the kidney for a given period of time, and then let the blood come back, there is a massive coagulation necrosis of tubules, proximal convoluted tubules.

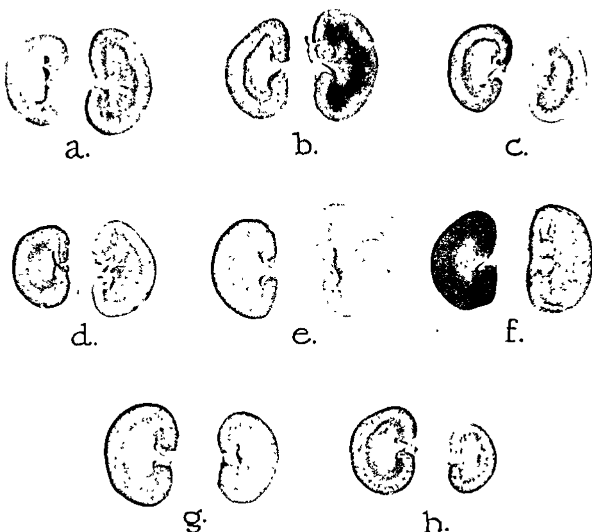


Fig 39. Gross appearance of injured left kidney at intervals after a two hour period of complete ischemia. The pairs of kidneys represent the injured left organ (on the right side) and the right kidney of the same animal as control. Variation in size of the control kidneys is due largely to difference in magnification: (a) 1 hour; (b) 6 hours; (c) 12 hours (the left kidney is enlarged, and the right kidney is hyperemic); (d) 24 hours; (e) 5 days (note pallor of both cortex and medulla, together with indistinct structure in e), (f) 1 week (the appearance of the kidney is that of an enlarged nephrotic organ), (g) 2 weeks, and (h) 3 weeks (note progressive reduction in size of kidney).

I call your attention to several distal convoluted tubules which are completely preserved. The glomerulus is bloodless to be sure, but its essential structure is preserved, but this massive coagulation has occurred in the proximal tubules. Evidently during the period of complete interruption of flow, there are profound chemical changes in the cells, but you have to let the blood come back in order for the phenomenon of coagulation necrosis to occur.

Now, it is perfectly obvious this picture does not even remotely resemble that of lower nephron necrosis, and there seems to be little doubt that episodes of complete interruption of blood flow to the rat kidney will not give a lesion that is morphologically like that of so-called lower nephron nephrosis.

It is true some investigators have pointed out that there are probably glomerular and proximal convoluted tubular components to what has been called lower nephron nephrosis Oliver suggested that the term ischemuric tubulorhexis be taken on to replace lower nephron nephrosis because the lesion is not really fixed to the distal convoluted tubule. It occurs in other parts of the nephron, and this particular term would give the proper emphasis to the various items of significance here like the ischemia and the anuria and the fact that the necrotizing lesion is partly connected with disruption of the tubules

But there is no morphologic evidence when you do the experiment this way that reduction in blood flow picks out the distal part of the nephron. This part seems spared, morphologically, in comparison with the proximal nephron The glomeruli, I think, are actually rendered more permeable because while it isn't shown in Figure 40, many of the capsular spaces contain protein exudate and casts of protein type are found within the collecting tubules

Now, this experience is quite different from what Oliver reported because he stated that in studying the dogs to which the Van Slyke group had applied renal clamps, he noticed that the lesion was isolated to the distal part of the nephron. He used the maceration and dissection technique, and suggested that the paraffin method is not sufficiently accurate to localize the lesion

Figure 41 shows repair of the necrotic lesion.

The debris is removed from the tubules, (Fig 42) and finally, they shrink down.

In Figure 43, the dwarfed kidney is apparent and it remains so with relatively small tubules, and reduced glomeruli There is a little interstitial reaction

This small kidney, we found, has a remarkable capacity of itself to undergo enlargement (Fig 44). You can show this very easily by taking out the kidney that hasn't been subjected to ischemia In other words starting with the small kidney, you can force it to function or kill the animal in renal failure.

When the opposite kidney is resected, the tiny, dwarfed kidney enlarges progressively, and becomes a big kidney and supports the animal, so it must undoubtedly function. However, there is an important time element here.

With a two hour period of ischemia, the above is the sequence of events. With a period of three hours of previous ischemia, progressive enlargement does not occur, only partial enlargement of the nephrons. With four hours of complete ischemia, nothing happens. The kidney stays a tiny kidney. It has no compensatory reaction, and the animal dies in uremia in about 3 or 4 days.

Figure 45 illustrates good compensatory hypertrophy where the glomerulus and all of the tiny tubules have become enlarged following the two-hour ischemic period and later resection of the normal kidney

In the average three-hour ischemia (Fig. 46) where one gets instead of a large, smooth kidney, a granular kidney, and only about half of the nephrons undergo compensatory enlargement

Microscopically, (Fig. 47), many of the tubules remain small while some are enlarged and hypertrophied In other words, the capacity for regeneration has a direct bearing on the time of the initial period of total ischemia.

Now, in a rat in which one kidney has had a two-hour or three-hour period of ischemia, if one removes the opposite normal kidney, the blood pressure rises rapidly and hypertension develops.

This is a phenomenon of great interest because while this tiny kidney and the normal kidney were in the body, the pressure was normal.

Blood pressure rises, although morphologically it is impossible to demonstrate that anything more is occurring in the ischemic kidney than that individual nephrons are undergoing progressive enlargement due to compensatory change. The heights of pressure vary, and it is hard to predict just how fast it will go up, whether it is a week or two, but the tendency is for it to go up. Of course, this phenomenon greatly interests us because this certainly looks as though the mechanism of hypertension here is due to withdrawal of the influence of a certain bulk of normal kidney substance.

After the resection of the normal kidney, the pressure is rising at a time when there is very little or no significant renal disease So the question then involves interpretation as to whether the withdrawal of the influence of the normal kidney is significant or whether the process of hypertrophy of tissue previously injured by ischemia is actually responsible for the hypertension, let's say, by elaboration of a specific pressor substance.

We also found that the ischemic kidneys which have very little in the way of glomerular disease at the beginning go on to develop diffuse lesions and the rats eventually die in uremia

These lesions, I think, are not dissimilar from human glomerulonephritis, with deposition of fibrinoid material, a marked capsular reaction, lipoid deposits, progressive fibrosis, and hyalinization of the glomerulus.

Why an animal develops lesions of this kind is of great interest.

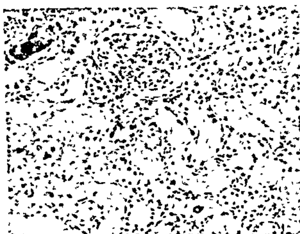


Fig. 40. Coagulation necrosis of proximal renal tubules 48 hours after a two-hour period of complete ischemia. Hematoxylin and eosin; X 158.



Fig. 41. Repair of tubular necrosis four days after a two-hour period of complete ischemia. Hematoxylin and eosin; X 158.

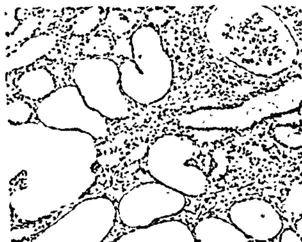


Fig. 42. Removal of necrotic debris one week after a two-hour period of complete ischemia. Hematoxylin and eosin, X 158.

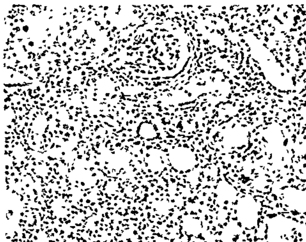


Fig. 43. Tubular atrophy three weeks after a two-hour period of complete ischemia. Hematoxylin and eosin; X 158.

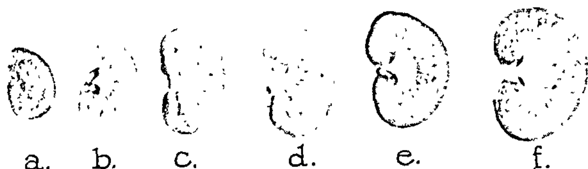


Fig. 44. Compensatory enlargement of atrophic left kidney at intervals after resection of opposite kidney; (a) one day, (b) three days, (c) one week, (d) two weeks, (e) three weeks, (f) one month. Note progressive increase in size. Renal circulation initially interrupted for two hours.

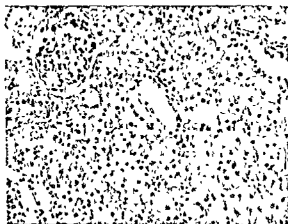


Fig. 45. Enlargement of glomeruli and tubules one month after resection of opposite kidney. Renal circulation interrupted for two hours. Hematoxylin and eosin; X 158.



Fig. 46. Partial compensatory enlargement of atrophic left kidney one month after resection of opposite kidney. Renal circulation interrupted for three hours.

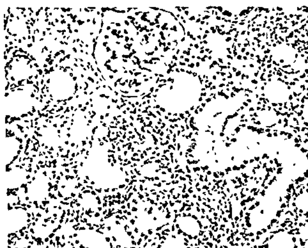


Fig. 47. Hypertrophied and atrophic tubules in kidney with partial compensatory enlargement one month after resection of opposite kidney. Renal circulation interrupted for three hours. Hematoxylin and eosin; X 158.

CHAIRMAN RILEY: Any questions?

DR. WALLACE McCRORY: What were the levels of BUN in the rat? Did you have them after you resected the weak kidney?

DR KOLETSKY: This again depended on the duration of the initial ischemia so that after a two-hour period, the levels were practically normal. After a three-hour period, they were slightly or moderately elevated -- 80 or so. After a four-hour period, they went progressively up, and the rat died in uremia.

DR. THOMAS: Is the little kidney functioning before you take out the good one?

DR KOLETSKY: I don't know. We have not determined whether it is functioning.

DR THOMAS: Is it making any water?

DR KOLETSKY: I don't know.

DR BARNETT. Have you by any chance tried the observation on hypophysectomized rats?

DR KOLETSKY: No

DR MAX MILLER· My question is the same Usually this whole proposition is seen better after a three-hour period of ischemia where the compensatory hypertrophy will just involve roughly 50% of the nephrons.

DR KOLETSKY In this instance the BUN is slightly to moderately elevated to levels of about 80, and these animals are hypertensive

CHAIRMAN RILEY: Do these rats with the hypertension die early? Do they have a pretty short life?

DR KOLETSKY: That's another variable thing. We have had some that died at about one to two months, and we had some that we followed for one to two years

They may live a short time or a long time This is one of the drawbacks in using this technique as a method of experimental hypertension. There is nothing wrong with it, but if you want to do chronic studies, all your animals may have hypertension and die early There is no way to predict it.

DR. JACK METCOFF. Is there anything unusual about what the rats are fed or how they eat?

DR KOLETSKY. Our rats were on ordinary dog chow I think that their food consumption is about average We did not make any quantitative measurements of how much they ate, but their weight curves are reasonable. Even those rats that are hypertensive and are, in fact, perhaps slightly uremic, seem to maintain a sort of plateau weight or perhaps increase very slightly, much less than a normal animal would do,

but they don't go downhill until they are about ready to die

DR NORMAN KRETCHMER: I would like to say a few words about Dr. Oliver's experiments. When he reviewed the kidneys, he showed quite clearly that the ischemia in the crush syndrome effectively was random in all portions of the tubule, but that the greatest effect was in the proximal tubule. He then went on with his dissection technique to clarify the situation of so-called lower nephron nephrosis. His feeling on this subject is that if one takes a three-dimensional section of a nephron, one can better predict whether the lesion is proximal or distal. The great failure in the beginning, after Bywaters' description of the crush syndrome, was that from flat, two-dimensional sections predictions were made

With the three-dimensional technique, it was obvious that ischemia was not only random, but predominantly proximal

DR. KOLETSKY: Oliver received for study dogs that had been subjected to a temporary period of complete ischemia, exactly as I did with the rats.

This was a standard procedure All animals were treated alike. They were all clamped completely for three hours, and then the clamp was released. However, the resulting picture, according to Oliver, varies greatly He said that proximal convoluted tubule lesions are found in some instances. In other instances, they are combined with distal tubular lesions. In other instances, he found what is called pure tubulorhexis, i.e , pure lower nephron nephrosis

There doesn't seem to be any degree of uniformity in the findings and the thing that bothers me here is that he concluded, therefore, that the reason for the variation was that there was a difference in the amount of blood that was actually circulating through the kidney or getting into the kidney.

It is difficult to see how, with a very standard procedure, all these variables occurred In other words, the implication is that the one lesion is due to incomplete ischemia and the other lesion is due to complete ischemia. The only possible way to explain this is on the basis of difference in collateral circulation in dogs, following these procedures, and in the rats, of course, I don't think this occurs because in experiments that are done by throwing something across the whole hilum, one doesn't have any collateral circulation

DR. KRETCHMER. Oliver not only studied dogs, but he collected human kidneys from all over the world. He received kidneys from Bywaters, where Bywaters originally described the crush syndrome. Kidneys were collected from the United States Army throughout the world where bombings were going on He defined the crush syndrome. He first named it acute renal failure, and the acute tubular necrosis was divided into two phases: The ischeminuric episode, the first phase in which there was an ischemia, and random destruction of the tubule, and in the second phase, if destruction went too far, there would be a lack of regeneration, was called tubulorexis, an actual destruction of proximal tubule cells.

CHAIRMAN RILEY: Thank you, Drs Thomas and Koletsky, for this stimulating presentation of your studies on experimental renal vascular disease

IV RENAL MECHANISM OF HYPERTENSION

CHAIRMAN JACK METCOFF: The program this morning will start with a consideration of the Renal Mechanism of Hypertension by Dr. Goldblatt and Dr. Haas

DR HARRY GOLDBLATT: It seems to me that the subject of hypertension has been more or less dragged into this conference, because hypertension is certainly not a usual accompaniment of nephrosis In my opinion, about the only justification for including hypertension in these discussions is that in the pathogenesis of both conditions the kidney plays an important part. Insofar as hypertension is concerned, this is perhaps best illustrated by the classification of this condition which is given in Table 9.

In this list of pathological conditions, usually associated with hypertension, the involvement of the kidney is common and, for some, glomerulonephritis, for example, the primary part played by the kidney in the pathogenesis of the associated hypertension is always conceded About the only types of hypertension in which the kidney probably plays no part are those which are associated with some form of endocrine disturbance (thyroid, adrenal, pituitary) and in the systolic hypertension of the aged It is my contention that in the other conditions, including so-called essential hypertension, the involvement of the kidney determines the elevation of the blood pressure.

I included essential hypertension because it is a fact that, although there may be no disturbance of renal excretory function, yet, almost invariably, vascular disease of the kidney is found in this condition. The rest of what I shall have to say in these introductory remarks will deal with the results of the attempts to prove the probable primary relationship between disease of the kidney, especially vascular disease of the kidney, and human essential hypertension It would take a long time, too long for this occasion, to tell the whole story Fortunately, with this group, I do not have to go into great detail, because you are undoubtedly well informed on the subject

We did not attempt to reproduce the intrarenal lesions of arteriolar nephrosclerosis. This has not been accomplished to date We retreated at once to the main renal artery, and, with the thought in mind that the probable hemodynamic disturbance in the kidney as a result of obliterative intrarenal vascular disease might, in the final analysis, be the cause of the elevated blood pressure, we constricted the main renal artery, with the hope that this might simulate the intrarenal circulatory disturbance.

TABLE 9

CLASSIFICATION OF TRUE HYPERTENSION
(according to associated conditions)

1 ESSENTIAL HYPERTENSION
(*Arterial and arteriolar nephrosclerosis*)

2. DIFFUSE GLOMERULONEPHRITIS

3 PYELONEPHRITIS CONTRACTED KIDNEYS

4. AMYLOID CONTRACTED KIDNEYS

5. RENAL ARTERIAL OBSTRUCTION

6 POLYCYSTIC RENAL DISEASE

7. PERIARTERITIS NODOSA

8 ADRENAL AND PITUITARY TUMORS

SYSTOLIC HYPERTENSION

1. THYROID HYPERPLASIA OR ADENOMA

2 *AORTIC AND LARGE VESSEL SCLEROSIS*

Surprisingly enough, the blood pressure rose, even when only one main renal artery was constricted In the dog, it remained up only 4 to 6 weeks, but in other animals it remained elevated for months. The removal of the clamp, or the excision of this kidney, resulted in a prompt fall of the blood pressure to normal. It was this observation which led later to the recognition of the occurrence in man of hypertension associated with unilateral renal disease, usually pyelonephritis and the associated obliterative vascular disease, and the cure of at least 100 individuals with this condition as a result of the removal of the one diseased kidney.

In the animals, to produce persistent hypertension, it was necessary either to *constrict the main renal* artery of one kidney and remove the other kidney, or to clamp both main renal arteries.

As a basis for the experimental work there were two working hypotheses. One was that, as a result of the constriction of the renal artery, the blood pressure would go up, and the other was that there would be no significant disturbance of renal excretory function as a necessary accompaniment of the hypertension. The results of our investigations showed definitely that both *hypotheses were completely satisfied. Significant* disturbance of renal excretory function was not a common accompaniment of this type

of hypertension. Of course, when both renal arteries were constricted to excess, or one main renal artery was greatly constricted and the other kidney was removed, then the renal excretory function did fail, the animals developed uremia and died within a week or 10 days. In such animals the typical necrotizing vascular lesions of the malignant phase of essential hypertension in man were exactly duplicated.

Much work was then done by ourselves and many other investigators on the pathogenesis of experimental renal hypertension. I cannot possibly go into the details of this phase of the work. As a result of many investigations done by many workers, particularly by Dr Page and his group, present here today, we have learned a lot about the pathogenesis of experimental renal hypertension. In the few moments left for my part of this discussion I can best epitomize in the next two tables the results of most of the investigations on what proved to be the humoral mechanism of experimental renal hypertension (Table 10) about which Dr. Haas will speak in detail, and in Table 11, give a comparison of what is known about human essential hypertension with the known facts about experimental renal hypertension

As I look at these tables again, I am impressed, as I always have been, with the close similarity between experimental renal hypertension and essential hypertension. I am reminded that when I was in high school and the Chemistry professor first discussed the matter of identification of various chemicals, he stated that "if a thing looks, smells, feels and acts exactly like something else, and if it has all the other properties of a known substance, then it is highly probable that it is that substance " This is the kind of reasoning I have used here in trying to induce you to believe with me in the probable renal origin of human essential hypertension

CHAIRMAN METCOFF· Dr. Haas will continue

DR. ERWIN HAAS· After the preceding discussion by Dr Goldblatt it may be understandable that the investigative work of our laboratory is centered pretty much around the kidney It involves in particular, at present, the isolated components of the humoral mechism of renal hypertension, shown in Figure 48. Renin, the enzyme in the kidney [1], reacting with hypertensinogen, the substrate in the plasma, forming hypertensin, which in turn exerts its hypertensive effect by constriction of the arterioles [2,3]

Of the experimental results discussed here, some will be presented with certain reservations imposed by the fact that the isolation of the individual components from the kidney requires the disintegration of the kidney structure While the pressor activity of these isolated, purified components are real, and can be demonstrated in bio-assays on normal animals, there seems at present no way to extrapolate and to evaluate the state of their activity in the intact kidney.

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[1] Tigerstedt, R and Bergman, P. G., Skand. Arch. F Physiol., 8. 223, 1898.
[2] Braun-Menendez, E , Fasciolo, J. C., Leloir, L F , and Munoz, J. M., Am. J. Physiol. 98 283, 1940
[3] Kohlstaedt, K G., Page, I H , and Helmer, O. M., Am Heart J., 19: 92, 1940.

Organ or Condition Studied	*Effect of Intravenous Injection of Renin in Normal Animals*	*Parallel in Experimental Renal Hypertension*
Heart Rate	Unaltered from normal [79 92,93]	Unaltered from normal [20]
Cardiac Output	Unaltered from normal [88 125]	Unaltered from normal [20]
Complete Sympathectomy	Does not reduce B P. rise due to renin [126]	Does not reduce B P. rise in experimental renal hypertension.[1,23,62 63]
Pithing	Does not reduce B P rise due to renin [79 92 93]	Does not abolish experimental renal hypertension [60 61]
Hypophysectomy	Does not reduce B P rise due to renin [109,122 127]	Does not prevent or abolish experimental renal hypertension [128,129]
Thyroidectomy	Does not reduce B P rise due to renin [127]	Does not prevent or abolish experimental renal hypertension [52]
Gonadectomy	Does not reduce B P rise due to renin [122]	Does not prevent or abolish experimental renal hypertension [130]
Acute Adrenalectomy	Does not reduce B P rise due to renin [93]	Does not reduce rise of B P when ischemic kidney of hypertensive dog is grafted into neck of normal dog [131]
Chronic Adrenalectomy	Abolishes B P response to renin [122,123 124]	Prevents or abolishes experimental renal hypertension [1,6,39 130,132]
	Response restored by adrenal cortical extract or D C S [124]	Hypertension maintained in adrenalectomized dogs treated with adrenal cortical extract or D C S [39]
Bilateral Nephrectomy	Response to renin greater [79 127,133 134 135]	Response to grafting of ischemic kidney of hypertensive dog greater in nephrectomized than normal recipient [8]
Peripheral Blood flow	No decrease during rise of B P due to renin [88]	No decrease in hypertensive rabbits with renal ischemia [29]
Renal Hemodynamics	Direct evidence shows decrease of renal blood flow [89 92 136]	Direct evidence shows decrease of renal blood flow [34]
	Indirect evidence of efferent arteriolar constriction [89]	Indirect evidence of efferent arteriolar constriction [159]
Blood Pressure	Infusion of renin causes persistent elevation [137]	Constriction of renal arteries causes persistent elevation [1]
	Rise not reversed by 933F [106,107]	The rise of B P due to release of the pedicle of a completely ischemic cat kidney is not reversed by 933F [107,118]
	Repeated injections cause tachyphylaxis [79,92 93 99]	If a cat is first rendered tachyphylactic to renin, release of pedicle of completely ischemic kidney is not followed by usual rise of B P [118 121]
	Renin causes no rise in pulmonary arterial pressure [138]	No rise of pulmonary arterial pressure in experimental renal hypertension [43]

D C S = Desoxycorticosterone acetate
B P = Blood pressure

TABLE 10. Reproduced from STUDIES ON EXPERIMENTAL HYPERTENSION. XVIII. Experimental Observations on the Humoral Mechanism of Hypertension by Harvey H. Lewis and Harry Goldblatt, Bulletin of the New York Academy of Medicine, Vol. 18, No. 7, pp. 459-487, July, 1942.

Subject	*Human Essential Hypertension*	*Experimental Renal Hypertension*
Cardiac rate	Normal	Normal
Cardiac output	Normal [17,19]	Normal [20]
Blood volume	Normal [21,22]	Normal [23,24]
Viscosity	Normal [25]	Normal [26]
Peripheral blood flow	Normal [27,28*]	Normal [29]
Sympathectomy	Does not abolish hypertension [27]	Does not prevent or abolish hypertension [7,23,62,63]
Resection of splanchnic nerves	Does not abolish hypertension [30]	Does not prevent or abolish hypertension [31]
Renal blood flow	Apparently reduced [32,33]	Reduced [34**]
Renal excretory function	(a) Benign phase normal [35,36]	Benign phase normal [1,3,5,6,24,37]
	(b) Malignant phase reduced [53]	Malignant phase reduced [39]
Cardiac hypertrophy	Left ventricle when uncomplicated by failure [40]	Left ventricle Rat,[41,42] Rabbit,[15] Dog [3,14]
Pulmonary arterial pressure	Not altered when hypertension is uncomplicated by left heart failure, as indicated by normal right heart [39]	Unaltered [43]
Unilateral renal disease associated with hypertension	Cured by nephrectomy when proved unilateral [44]	Cured by nephrectomy [3,5,6,45]
Bilateral nephrectomy	No rise of pressure [46]	No rise of pressure [7,47,50]
Thyroidectomy	Does not prevent or cure hypertension unless of the type associated with disease of the thyroid [51]	Does not prevent or abolish hypertension [52]
Generalized arteriolar necrosis and necrotizing arteriolitis	In malignant phase only [38,53,54]	In malignant phase only [55,57]

* Controversial Abramson, D I and Fierst, S M Resting blood flow and peripheral vascular responses in hypertensive subjects, *Am Heart J*, 1942, 23 84

** Controversial Corcoran A C and Page, I H Renal aspects of experimental and clinical hypertension, *J Lab & Clin Med* 1941, 26 1713

TABLE 11. Reproduced from STUDIES ON EXPERIMENTAL HYPERTENSION. XVIII. Experimental Observations on the Humoral Mechanism of Hypertension by Harvey H. Lewis and Harry Goldblatt, Bulletin of the New York Academy of Medicine, Vol. 18, No. 7, pp 459-487, July, 1942.

A procedure was devised for the isolation of renin in a highly purified form and on a large scale [4], and an outline of the experimental conditions is shown in Table 12.

TABLE 12

ISOLATION OF RENIN FROM HOG KIDNEYS

Isolation of Renin	mg. Protein / Unit Renin	Purification
Extract of Kidney Tissue	71	1
Autolysis, Benzene, pH 8 5, 38°C.	18	4
Ethanol Fractionation at pH 2.1	1 1	65
Sedimentation of Renin - Tungstate		
$(NH_4)_2SO_4$ - Fractionation pH 4 3	0.28	260
Acetone " " 4 8	0 10	670
$(NH_4)_2SO_4$ " " 5 5	0.036	2,000
" " " 7 5	0.021	3,400
" " " 3 9 25°C.	0 009	8,000
" " " 2.5	0.005	13,000
Acetone " " 3 5	0.0013	56,000

As a result of the purification it can be seen that about 1/1000 of one mg of protein *will yield one unit of renin, in contrast to 71 mg. of substance required in the* form of the crude first extract of kidney tissue. Hog renin was administered to human hypertensive patients over long periods and in repeated doses for the production of antirenin [5, 6] Some of the patients received 40-60 injections in an 8 months' immunization period, each dose of 4,000 units representing an extract of about 3,000 gm. of foreign kidney substance. It is obvious, therefore, that the purification of the renin represents an essential prerequisite to avoid undesirable side reactions typical for foreign proteins, i.e , anaphylactic shock, blood pressure depression, interference with the clotting mechanism, etc

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[4] Haas, E., Lamfrom, H., and Goldblatt, H., Arch Biochem. and Biophys , 42: 368 1953

[5] Goldblatt, H , Haas, E. and Lamfrom, H., Trans. Assoc Am Phys. 64. 122, 1951.

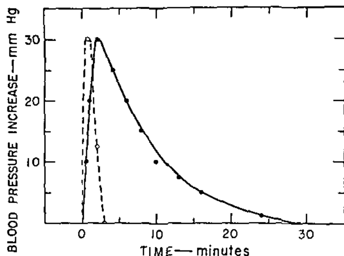
[6] Lamfrom, H., Haas, E. and Goldblatt, H., Am. J. Physiol., 177: 55, 1954.

HYPERTENSINOGEN

RENIN

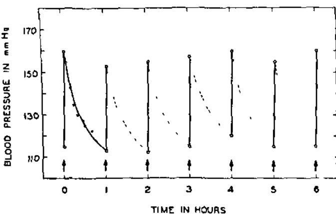
HYPERTENSIN

✓Fig. 48. Humoral mechanism of renal hypertension



Solid line. 1 unit renin
Dotted line. 1 unit hypertensin

✓Fig 49 Blood pressure elevation after injection of renin and hypertensin



✓Fig. 50. Blood pressure response to repeated injections of renin

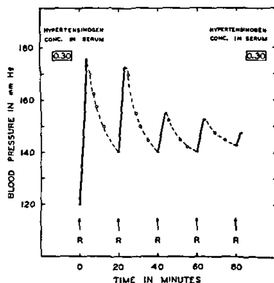


Fig. 51. Development of tachyphylaxis in an anesthetized dog

The purity of the new renin preparation, relative to that obtained by previous methods, is illustrated in Table 13 Also compared in the same illustration are the amounts of kidney tissue which can be processed by the new procedure, another essential factor in obtaining sufficiently large quantities of renin for its possible application to patients.

One of the tools employed in this type of study is a special construction of a hydraulic press which permits the processing of 150 pounds of kidney in a few hours.

TABLE 13

SPECIFIC ACTIVITY OF VARIOUS RENIN PREPARATIONS
AND AMOUNTS OF KIDNEYS PROCESSED

Comparison with Previous Methods

Authors	Year	Kg. Kidneys	Units Renin / Mg Nitrogen
Helmer and Page	1939	1	3
Collings, Remington et al.	1940	3	6
Schales	1942	1	17
Katz and Goldblatt	1943	4	130
Marshall and Wakerlin	1949	10	125
Haas, Lamfrom, Goldblatt	1951	70 - 140	5,000

A rapid and accurate bio-assay is available for renin [7] and for hypertensin [8] and this has been of considerable importance in a variety of studies. It served as a guide in the isolation procedure for renin, in the detection of undesirable substances, in measuring the renin concentration in the constricted kidneys of hypertensive animals, and it was employed in immunological studies concerned with the formation and action of antirenin The intravenous injection of renin or hypertensin into normal, trained, unanesthetized test dogs induces an elevation of the mean femoral blood pressure of the animal as shown in Figure 49.

The elevation of the blood pressure by hypertensin is rapid and of short duration. In contrast, more time is required for renin to exert its peak pressure and its effect on the blood pressure is much more prolonged The role of renin, as an enzyme, and of hypertensin, as the metabolic end product of the reaction, is reflected in their physiological action on the blood pressure

[7] Goldblatt, H., Katz, Y. J., Lewis, H. A. and Richardson, E., J. Exp. Med., 77: 309, 1943

[8] Goldblatt, H., Lamfrom, H , and Haas, E., Am J. Physiol., 175: 75, 1953.

DR. JANEWAY: If this were human plasma, what would you say are the limits of accuracy with this method?

DR. HAAS: Using average values obtained on three test dogs I would say that renin or hypertensin can be determined with an accuracy better than ± 10%.

DR. JANEWAY: Do you get tachyphylaxis?

DR. HAAS: There is no tachyphylaxis under the experimental conditions of our bio-assay procedure. However, conditions can be arranged at will, either to avoid tachyphylaxis or to induce this refractive state. As shown in Figure 50, a practically identical blood pressure elevation was elicited in each case, following the injection of 7 successive doses, of 2 units of renin, at intervals of 1 hour, into a normal dog. Tachyphylaxis does not occur under these conditions

In contrast, the blood pressure responses to equal quantities of renin decreased progressively when the dose was increased to 7 units and when the time interval between the injections was shortened to 20 minutes. This is shown in Figure 51. The hypertensinogen concentration in the serum of the dog remained unchanged throughout this procedure, so the diminished response to the injected renin cannot be ascribed to a depletion of the renin substrate in the test animal.

DR GOLDBLATT. I think the main reason why we do not get tachyphylaxis is we rarely use the same animal twice in the same day, for a renin determination, and we never use an animal for two consecutive tests for renin on any day.

DR. JANEWAY: You can use dogs repeatedly, for a long time?

DR. GOLDBLATT: We have used some for 8 years.

DR. HAAS: The pressor response of a normal dog to hog renin, as a function of time and of renin concentration, has been plotted in Figure 52 and it can be seen that the relative area under these curves represents a sensitive index of renin activity.

The peak of the blood pressure elevation after the injection of various amounts of renin is reached in 2 minutes and, as shown in Figure 53, this elevation is directly proportional to the amount of renin injected up to a rise of 35 mm. Hg. This represents the basis for the bio-assay of renin and the amount of renin that will raise the blood pressure of an unanesthetized trained dog 30 mm. Hg. has been designated as one unit.

The injection of a single dose of renin has been shown to induce only a temporary rise in blood pressure. If renin actually plays a part in hypertension, it should be possible to select experimental conditions under which it maintains the blood pressure continuously at an elevated level. Such conditions have been established in the experiments shown in Figure 54, by the continuous infusion of small amounts of renin.

✓The peak pressure in Figure 54 is maintained as long as the infusion of renin is continued and persists for some time after it is stopped. This experimental arrangement might be of interest also for the evaluation of hypotensive drugs.

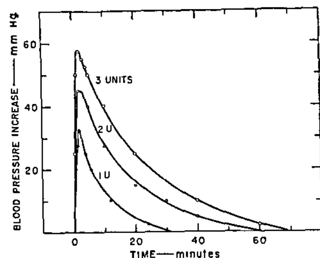


Fig. 52 Blood pressure response to renin as function of time

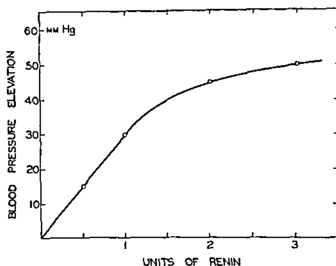
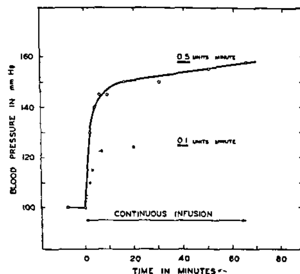
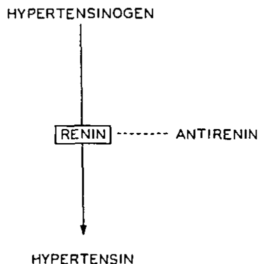


Fig 53. Increase of blood pressure as a function of renin concentration



✓Fig 54 Blood pressure response to continuous infusion of renin



✓Fig 55. Antirenin in the humoral mechanism of renal hypertension

The chemical nature of renin as an enzyme and protein suggested that its injection into experimental animals, and subsequently into hypertensive patients, might result in the immunological formation of antirenin [9, 10, 11, 6], corresponding to the inhibition of an enzyme by its anti-enzyme, a neutralization of the renin activity by antirenin could be anticipated, and it was hoped that this might provide the basis for a therapeutic treatment of human hypertensive patients, especially after renin had become available in a highly purified form (Fig. 55).

It was soon established that the immunized animal became completely unresponsive to renin, even to the intravenous injection of very large amounts, such as 100 units [6], and this was the basis of our attempt to produce antirenin in human patients.

The presence of antirenin in the serum of an immunized animal or patient can be determined quantitatively by permitting its instantaneous reaction, the inactivation of renin, to take place at room temperature, in-vitro Any residual renin remaining thereafter in its active form is determined, subsequently, by the injection of the renin-serum mixture into test dogs, in the usual bio-assay for renin. The amount of antirenin which will inactivate one unit of renin under these conditions has been designated as one unit Testing at the 10-unit level, the titration of antirenin can be performed within 5% of accuracy, an unusually high degree, compared to customary immunological procedures This permits immunological studies of a perhaps more general interest, i e , on the dissociation of the antigen-antibody complex, on the chemical properties of the antibody, on the specificity of the renin-antirenin interaction, etc.

With this method it was demonstrated [6] that the enzymatic activity of renin is not neutralized or blocked by its interaction with antirenin but that it is irreversibly destroyed. Following its reaction with renin, antirenin dissociates from the complex under a variety of conditions (Table 14), even spontaneously, under physiological conditions Thus antirenin becomes available again to interact with additional amounts of renin, almost in a catalytic fashion, in contrast to the customary concept of a stoichiometric antibody-antigen relation.

The amounts of renin and the time required for the active immunization of human, dog and rat are shown in Table 15, together with the maximum antirenin titers which were attained, finally, in their sera

The patients were usually injected with 4,000 units of renin, intramuscularly, twice a week

DR JANEWAY: Hog renin?

DR HAAS Yes, but purified about 2,000-fold in the first 6 steps of the new isolation procedure [4] There is a very pronounced immunological response of the

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[9] Johnson, C A and Wakerlin, G.E., Proc Soc. Exper Biol. and Med , 44: 277, 1940.
[10] Goldblatt, H , Katz, Y J , Lewis, H. A., Richardson, E., Guevara, Rojas, A., and Gollan, F , Proc Central Soc Clin. Research, 15. 31, 1942.
[11] Helmer, O M., Shipley, R E , Peirce, J. D., and Kohlstaedt, K. G., J. Lab. and Clin Med , 33: 1484, 1948

patients or experimental animals, to renin, i.e., the amount of antirenin formed is large, considering for example, that each cc. of the patient's serum contained sufficient antirenin to inactivate 37 units of hog renin.

TABLE 14

RECOVERY OF ANTIRENIN FROM ITS COMPLEX WITH RENIN

Conditions for Splitting Complex	Recovery of Renin %	Recovery of Antirenin %
1) pH 11 3	0	90
2) 3 M NaCl	0	80
3) Dialysis	0	52
4) Saline 20 hrs.	0	50

TABLE 15

ANTIRENIN FORMATION FOLLOWING INJECTION OF RENIN

	Total Amount Injected	Immunization Period	Antirenin Titer
	units renin	weeks	units / cc serum
HUMAN	112,000	20	37
DOG	15,000	31	20
RAT	10	2.5	25

DR. JANEWAY: Do they develop local reactions to the injection during this immunization?

DR. GOLDBLATT No reaction, or only slight erythema at the site of injection.

DR. HAAS: The experimental results obtained in the rat are of practical interest: In contrast to the requirements of man and dog, considerably smaller amounts of renin are sufficient to induce, in a short time, the formation of high concentrations of antirenin.

The high degree of antigenicity of renin becomes apparent by comparing it, in terms of mg. of substance required for immunization, with other enzymes such as catalase, urease, etc. This is shown in Table 16.

TABLE 16

COMPARISON OF RENIN WITH OTHER ENZYMES

	Total Antigen Injected mg.	Inhibitign of Enzyme %	Enzyme-Antienzyme Complex
RENIN	*0 01*	*100*	*soluble*
CATALASE	100	0	ppt.
UREASE	30	30 - 80	ppt.
LACTIC ACID DEHYDROG.	120	10 - 75	

The catalytic activity of renin is completely abolished by antirenin, as compared in Table 16, with other enzymes which remain partly or completely active in the presence of their anti-enzymes. The renin-antirenin reaction proceeds without the formation of a precipitate, in contrast to the insoluble precipitates formed by the other enzyme-anti-enzyme complexes shown in Table 16. All of these are desirable features and necessary prerequisites for the application of renin in the immunological treatment of hypertension. In Table 17 are shown the amounts of renin administered to a number of patients and the antirenin titers obtained after various periods of immunization.

TABLE 17

ANTIRENIN FORMATION IN HUMAN HYPERTENSIVE PATIENTS

PATIENT	TREATMENT Weeks	RENIN INJECTED Units	ANTIRENIN Units cc Serum
Mrs. B.T.	3.5	28,000	0.8
Mr. H.W	5	20,000	0.4
Mrs L E S.	6	48,000	0
Mrs. B C.	7	56,000	11
Mr. V.M.	8	64,000	1.0
Mrs S.K	20	112,000	16
Dr I Y.B	20	132,000	37
Mr. H B	33	1421000	2.0

No significant lowering of the blood pressure could be observed, even in the hypertensive patients with high antirenin titers [5]. This may be attributed to the fact

that the antirenin to hog renin, developed in man did not block the activity of human renin, even in vitro and at a ratio of antirenin to renin of 60 to 1. The antirenin induced in man or dog by the injection of hog renin was found to inactivate, with various degrees of efficiency, renin of every animal species investigated, with the exception of human renin (Table 18). On the basis of these results, the application of antirenin to the therapy of hypertension has been discontinued, for the time being.

TABLE 18

CAPACITY OF ANTIRENIN TO NEUTRALIZE RENIN FROM VARIOUS SPECIES

ANTIRENIN TO HOG RENIN IN	RENIN FROM						
	HOG	DOG	RABBIT	BEEF	SHEEP	RAT	HUMAN
Dog Serum	1.0	0.5	0.4	0.3	0.1	0.1	0
Human "	1.0	0.7	0.2	0.2	0.1	0.1	0

A certain degree of unspecificity of antirenin to hog renin was revealed by the results shown in Table 18 Preliminary experiments, to modify the antigen, hog renin, chemically, were unsuccessful, so far, in an attempt to broaden the unspecificity of the antirenin produced to such an extent that it would react with human renin.

Quantitative considerations raise some questions as to the actual state of activity of renin in the intact kidney. The isolated renin, under physiological conditions, is effective in very small amounts, for example, the infusion of 0.1 units of renin per minute is sufficient to induce an appreciable blood pressure elevation in a normal dog (Fig. 54). On the other hand, about 1500 times as much renin can be obtained from the kidneys of a normotensive dog, suggesting that renin might not be accessible in the intact kidney, or that it might be present in an enzymatically inactive state.

An investigation of semi-purified renin preparations of all three species investigated, rabbit, dog and hog, revealed that a modification of their pressor properties can be induced, for example, by acidification This alteration of the pressor pattern of renin can be visualized by a comparison of the graphs in Figures 56 and 57.

The injection of semi-purified renin (Figure 56) can be seen to result in a delayed, prolonged blood pressure elevation, with a plateau-like peak pressure, and the rise in blood pressure does not reach its maximum at the 2 minute period. In contrast, aliquots of the same renin preparation, subjected to a brief period of acidification, induce a somewhat greater blood pressure elevation (Fig. 57) with the appearance of definite pressor maxima, 2 minutes after the injection

The renin content of the semi-purified preparation cannot be estimated from its pressor action, at the customary 2 minute period, because the response does not reach the maximum at this time, and it is not directly proportional to the amount of

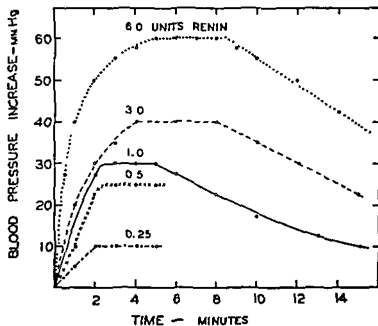


Fig. 56 Blood pressure response to semi-purified rabbit renin

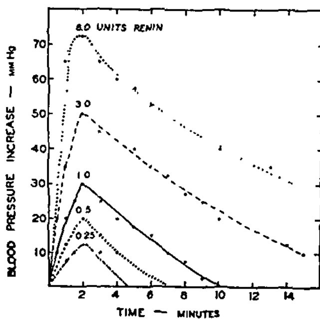


Fig 57 Blood pressure response to acid-modified rabbit renin

renin administered For example, the injection of an amount of the semi-purified preparation (Fig. 56) equivalent to 3 units of the more purified preparation induced a pressor response, at the 2 minute interval, corresponding to the blood pressor elevation induced customarily by only 1 unit of the more purified renin.

The increased blood pressure response, at the 2 minute interval, from the preparation after acidification, was previously evaluated erroneously as an activation of a precursor of renin In reality it indicates only a modification of the time-pressure pattern, with a shift of the pressure peak to the 2 minute period.

The different response of the test dogs to the two renin preparations of different degrees of purity cannot be ascribed to a foreign protein reaction, but it may be the result of the presence of impurities in the semi-purified preparation. Homologous dog renin, prior and after acidification, induces the time-pressure patterns identical with those of rabbit and hog renin.

The presence in the serum of increased amounts of hypertensin (Skeggs, Kahn et al. [12, 13]) suggested indirectly that an unusually high concentration of renin may be active in the kidneys of hypertensive patients and animals, catalyzing the reaction shown in Figure 48. If renin plays a part in the humoral mechanism of hypertension, a more direct determination of its concentration seemed to be of importance, especially under conditions in which a kidney with an impaired circulation can be implicated definitely as the cause of hypertension.

The results of such an experiment are shown in Table 19 in which the benign phase of experimental renal hypertension had been established by the partial constriction of the left main renal artery of 7 dogs [14, 15]

An elevation of the renin content is apparent in the kidney with the constricted circulation of all the hypertensive dogs, investigated 13-30 days after clamping, when the blood pressure of the animals had reached its maximum.

At the same time, when the concentration of renin was found to be abnormally high in the constricted kidney, the renin content of the unrestricted, opposite kidney, was reduced consistently to 1/6 of the values found in normal kidneys.

The average of 4 2 units/gm. in the clamped kidney and of 0.3 units/gm. in the contralateral, unrestricted kidney, represent very significant deviations from the normal renin content of kidney tissue (1/8 units/gm.)

While a mechanical clamp was employed in the preceding experiment, to restrict the renal circulation of dogs, it seemed as if a physiological interference with the

[12] Skeggs, L. T. Jr , Kahn, J R. and Shumway, N. P., J. Exp. Med., 95: 241, 1952.
[13] Kahn, J. R., Skeggs, L. T Jr , Shumway, N. P. and Wisenbaugh, P. E., J. Exp. Med., 95: 523, 1952
[14] Goldblatt, H., Lynch, J., Hanzal, R. F., and Somerville, W. W., J. Exp. Med., 59. 347, 1934.
[15] Goldblatt, H , Am. J. Clin. Path., 10 40, 1940.

renal hemodynamics might be established by the intramuscular injection of a single large dose of hog renin into the dog. This is indicated, in Table 20, by a prolonged elevation of the dog's blood pressure, following the injection of single doses, 25 to 350 units.

These injections, repeated twice weekly over a period of several weeks, induce the formation of antirenin in the dog and an increase in the renin concentration in the kidney of the animal to very high amounts [16], 14 times that of normal dog kidneys as shown in Table 21.

TABLE 19

RENIN CONTENT OF DOG KIDNEYS AFTER UNILATERAL CONSTRICTION

DOG	CONSTRICTION OF RENAL ARTERY	BLOOD PRESSURE		UNITS RENIN / GM KIDNEY	
		INITIAL	MAXIMUM	CONSTRICTED KIDNEY	OPPOSITE KIDNEY
1	13 DAYS	105	175	8.7	0.3
2	23 "	115	185	4.6	0.3
3	15 "	130	190	4.3	0.4
4	30 "	125	185	4.2	0.3
5	18 "	120	170	3.8	0.3
6	15 "	120	165	3.0	0.3
7	21 "	135	180	1.4	0.3
AV	19 DAYS	120	180	4.2	0.3

The formation of antirenin, and not the elevated blood pressure, appears to be the cause of the increased renin content of the kidney. This is suggested by additional experiments performed on rabbits and shown in Table 21.

1 The intraperitoneal injection of hog renin in the rabbit had no effect on its blood pressure, yet it induced the formation of antirenin, and it resulted in a 4-fold increase of the renin concentration in the kidney of the animal [6].

2 The injection of rabbit renin into rabbits did not lead to the development of antirenin, presumably because of the homologous nature of the antigen The renin content of the rabbit kidneys was actually depressed under these conditions, not elevated [6].

The experimental studies presented here may have very little bearing on the nephrotoxic syndrome, aside from the fact that both are concerned with the kidney. There may be common denominators, for example, the massive proteinuria encountered in the nephrotoxic syndrome can also be reproduced by the administration of renin [17].

[16] Becker-Brennan, B., Burns, R. O. and Wakerlin, G. E., Federation Proc., 12: 20, 1953.

[17] Addis, T., Barrett, E., Boyd, R. I., and Ureen, H J., J. Exp. Med., 89: 131, 1949.

TABLE 20

BLOOD PRESSURE ELEVATION OF DOGS
AFTER INTRAMUSCULAR INJECTION OF RENIN

TIME AFTER INJECTION	(25)	(100)	(230)	(350)
HOURS	BLOOD PRESSURE ELEVATION — MM Hg			
1	25	52	33	50
2	25	37	50	55
6	0	20	45	32
24		0	40	35
30			37	30
48			0	10
54				0

TABLE 21

CONCENTRATION OF RENIN IN THE KIDNEYS OF NORMAL AND IMMUNIZED ANIMALS

Species	Antigen Injected	Renin Content units/gm kidney
Dogs	-	1.8
"	Hog renin	25
Rabbits	-	11
"	Hog renin	40
"	Rabbit renin	4

renal hemodynamics might be established by the intramuscular injection of a single large dose of hog renin into the dog This is indicated, in Table 20, by a prolonged elevation of the dog's blood pressure, following the injection of single doses, 25 to 350 units.

These injections, repeated twice weekly over a period of several weeks, induce the formation of antirenin in the dog and an increase in the renin concentration in the kidney of the animal to very high amounts [16], 14 times that of normal dog kidneys as shown in Table 21.

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DOG	CONSTRICTION OF RENAL ARTERY	BLOOD PRESSURE		UNITS RENIN / GM KIDNEY	
		INITIAL	MAXIMUM	CONSTRICTED KIDNEY	OPPOSITE KIDNEY
1	13 DAYS	105	175	8 7	0 3
2	23 "	115	185	4 6	0.3
3	15 "	130	190	4 3	0 4
4	30 "	125	185	4 2	0 3
5	18 "	120	170	3.8	0.3
6	15 "	120	165	3 0	0 3
7	21 "	135	180	1 4	0 3
AV	19 DAYS	120	180	4 2	0 3

The formation of antirenin, and not the elevated blood pressure, appears to be the cause of the increased renin content of the kidney. This is suggested by additional experiments performed on rabbits and shown in Table 21.

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[16] Becker-Brennan, B., Burns, R. O. and Wakerlin, G. E., Federation Proc., 12: 20, 1953.

[17] Addis, T., Barrett, E., Boyd, R. I., and Ureen, H. J., J. Exp. Med., 89: 131, 1949.

TABLE 20

BLOOD PRESSURE ELEVATION OF DOGS
AFTER INTRAMUSCULAR INJECTION OF RENIN

TIME AFTER INJECTION	25	100	230	350
HOURS	BLOOD PRESSURE ELEVATION — MM Hg			
1	25	52	35	50
2	25	37	50	55
6	0	20	45	32
24		0	40	35
30			37	30
48			0	10
54				0

TABLE 21

CONCENTRATION OF RENIN IN THE KIDNEYS OF NORMAL AND IMMUNIZED ANIMALS

Species	Antigen Injected	Renin Content
		units / gm. kidney
Dogs	-	1.8
"	Hog renin	25
Rabbits	-	11
"	Hog renin	40
"	Rabbit renin	4

For this reason, a progress report on studies of the humoral mechanism of renal hypertension may, perhaps, be of some interest.

CHAIRMAN METCOFF: Thank you, Dr. Haas. Dr. Skeggs will continue.

DR. LEONARD SKEGGS, JR : The group at the Veterans Administration Hospital in Cleveland decided to determine whether hypertensin or angiotonin is the substance responsible for the elevation of blood pressure in experimental renal hypertension in animals as well as in human beings with either benign or malignant essential hypertension. In order to do this, it seemed necessary to prepare extracts of hypertensin from blood and concentrate and purify them so that they might be tested for pressor action in a small and sensitive animal.

Since hypertensin is a dialysable substance, it occurred to us that it could be separated from the blood by the use of an artificial kidney.

One of our first experiments [18] is illustrated in Figure 58. The artificial kidney [19] is connected to the femoral artery of a dog from which blood flows through the kidney and back to the animal via the femoral vein. While the blood is flowing between sheets of cellophane in the kidney, dialysate or salt solution is re-circulated on the opposite side of the membrane The animals were dialysed in this manner for 90 minute periods during which time 300 ml of dialysate was brought into equilibrium with the blood with respect to its hypertensin content

The 300 ml. samples of dialysate thus obtained were chemically purified and concentrated about 300-fold and assayed in the rat

The assay preparation [20] which was used is shown in Figure 59. A small bore polyethylene catheter is inserted into the left jugular vein to provide a method of injecting the extracts. A larger polyethylene catheter is inserted into the trachea to provide a free airway. Both vagus nerves are cut. The right carotid artery is cannulated and connected to a mercury manometer equipped for inkwriting on the kymograph.

Figure 60 is a typical result obtained by this assay method At 1, injections of 0.01 Goldblatt unit were given and at 2 and 3, respectively, 0.1 ml. and 0 14 ml. of an unknown solution were given It can be concluded from this assay that 0.12 ml. of the unknown solution was equivalent to 0.01 of a Goldblatt unit.

Applying these methods to normal dogs [21], we obtained the results shown in Table 22. It is evident from this table that the blood of normal dogs contained very little hypertensin.

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[18] Kahn, J. R , Skeggs, L T., Shumway, N. P., Circulation, 2: 363, 1950.
[19] Skeggs, L. T , Leonard, J R., Heisler, C. R., Proc. Soc. Exper. Biol. Med. 72: 539, 1949.
[20] Skeggs, L. T., Kahn, J. R., Marsh, W. H., Lab. Investigation, 2: 109, 1953.
[21] Skeggs, L. T., Kahn, J. R., Shumway, N. P., Circulation, 3; 384, 1951.

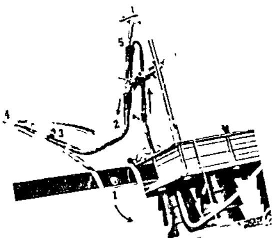


Fig 58 Illustrating application of artificial kidney

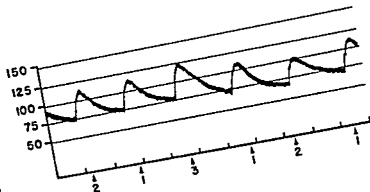


Fig 60 Typical assay curve comparing unknown dialysate to known strength hypertensin

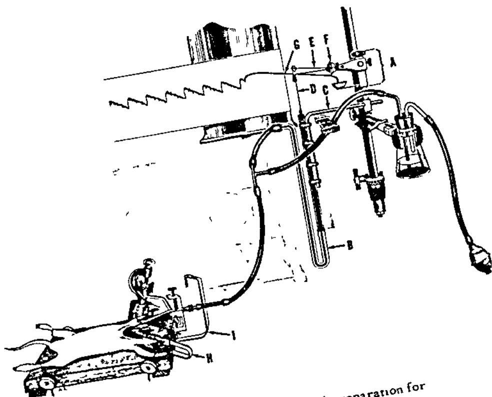


Fig 59 Experimental animal preparation for hypertensin assay

TABLE 22

NORMAL DOGS

Dog No.	Units of Hypertensin Per Liter of Dialyzate
34	0.04
37	0.03
38	0.08
39	0.08
45	0.00
42	0.00
32	0.00

TABLE 23

DOGS WITH BENIGN EXPERIMENTAL RENAL HYPERTENSION

Dog No	Mean Pressure	Operations	No. Days Hypertensive	Hypertensin U/L Dialyzate
42	140	LK	4	0.53
43	150	LK	6	0.41
32	200	LK & RK	7	0.66
28	165	LK & RK	10	0.52
29	175	LK	15	0.18
16	165	RK & LK	21	0.19
16	155	RK & LK	25	0 10
31	205	LK & RK	29	0 75
27	155	LK & RK	90	0.13

Similar experiments were performed on dogs with experimental reanal hypertension produced by the Goldblatt method. Dialyses were performed at varying periods after hypertension had developed and it is evident from the results in Table 23 that there is a marked increase in the amount of circulating hypertensin

✓Although these experiments were encouraging, we did not feel that it was practical to apply the artificial kidney to patients just for the purpose of determining whether they had hypertensin in their blood. We, therefore, developed a chemical method of obtaining hypertensin directly from blood [22]. By means of this method, shown in Table 24, we were able to concentrate the hypertensin from 250 ml. of arterial blood to a volume of 1 ml. This concentrate was not depressor, was isotonic with blood, and was suitable for assay in the rat.

[22] Skeggs, L. T., Kahn, J. R., Shumway, N. P., J. Exper. Med. 95: 241, 1952.

TABLE 24

'ISOLATION OF HYPERTENSIN'

	Discard
250 ml. Arterial Blood + 1 L 95% Alcohol Stir, filter, wash with 1 L 80% Alcohol	Precipitate
Filtrate acidified and evaporated to 100 ml. Centrifuge	Precipitate
Supernatant extracted with Ether	*Lipids in Ether layer*
Aqueous layer evaporated to 10 ml. Extract with n-butanol at pH 2.5	Aqueous layer
Butanol extract adsorbed on Al_2O_3 column	Butanol
Al_2O_3 column washed with 85% Alcohol	85% Alcohol
Al_2O_3 column washed with 50% Alcohol	Al_2O_3
50% Alcohol eluate evaporated to dryness and dissolved in 1 ml. at pH 7.3 and test biologically	

In order to test the ability of the method to recover hypertensin, 0.2 of a Goldblatt unit was added to 250 ml. samples of blood which were then processed by the chemical method just described. The overall recovery of hypertensin, as shown in Table 25, was about 50%.

A group of dogs was then bled and the samples processed by the chemical method shown in Table 24. Malignant hypertension was then produced in these animals and additional blood samples taken 48 and 72 hours later. The results are illustrated in Table 26. It can be seen that there is a marked increase in the concentration of hypertensin in the blood of these animals after their renal arteries had been constricted.

Although the amount of hypertensin obtained in dogs with experimental hypertension was greatly in excess of the amounts found in normal dogs, it appeared to us that the amounts were quite small and possibly ineffective in causing the elevation of blood pressure. We, therefore, devised experiments designed to show what the effective blood level of hypertensin must be in order to raise the blood pressure of dogs.

Two such experiments are shown in Figures 61 and 62. In Figure 61, hypertensin was infused During the time when the blood pressure was elevated, the amounts of hypertensin were found to be 0.16, 0 48 and 0.24 Goldblatt units per liter of blood. In Figure 62, renin was infused in place of hypertensin. Samples were taken at various intervals and it was found that while the blood pressure was elevated, 0.24, 0.54, 0.80, 0.68, 0.28 Goldblatt units of hypertensin were found per liter of blood. The animals used in these experiments had been nephrectomized so that the hypertensin recovered could not have originated in the animals' kidneys The large volumes of blood withdrawn

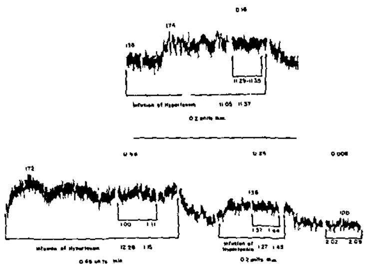


Fig 61 Effect of hypertensin level on blood pressure of nephrectomized normal dogs

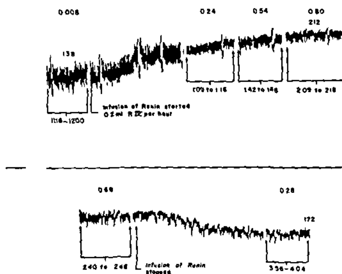


Fig 62 Effect of renin infusion on blood pressure of nephrectomized normal dog

from the dogs for the hypertensin samples were replaced by blood from bilaterally nephrectomized donor dogs.

TABLE 25

RECOVERY OF HYPERTENSIN ADDED TO BLOOD

Sample No.	Hypertensin added	Recovery of hypertensin	
	units	units	per cent
17 B	0 2	0 11	55
17 C	0 2	0.11	55
17 D	0 2	0.11	55
17 E	0.2	0.10	50
17 F	0.2	0 10	50
17 G	0.2	0.12	60

TABLE 26

Assay of Hypertensin from the Blood of Dogs before and after Constriction of the Renal Artery with Development of Malignant Hypertension

	Before constriction	48 hrs after constriction			72 hrs after constriction		
Dog No	Hypertensin found	Blood urea nitrogen	Mean femoral blood pressure	Hypertensin found	Blood urea nitrogen	Mean femoral blood pressure	Hypertensin found
	units per liter	mg per 100 ml	mm Hg	units per liter	mg per 100 ml	mm Hg	units per liter
43	0 03	75	185	0 10	104	190	0 10
67	0 04	190	190	1 2	Dead	Dead	Dead
41	0 02	96	165	0 20	207	155	0 25
55	0 05	150	160	0 17	146	175	1 0
62	0 02	146	175	0 12	Dead	Dead	Dead
76	No sample	No sample	185	0 20	218	165	1 6
80	0 05	104	155	0 28	159	175	0 30
82	0 02	93	185	0 24	153	190	0 28
83	0 04	86	165	0 29	141	155	1 0
89	0 02	119	160	0 10	150	70	0 21

Encouraged by these results, we proceeded to the assay of hypertensin in the blood of humans [23] The results obtained on a group of 24 normotensive patients are tabulated in Table 21 In half of this group we were unable to find any hypertensin. The others had extremely small amounts One patient has as much as 0 05 units per liter

[23] Kahn, J R., Skeggs, L. T., Shumway, N. P , Wisenbaugh, P. E., J. Exper Med. 95: 523, 1952.

TABLE 27

ASSAY OF HYPERTENSIN IN THE BLOOD OF NORMAL PATIENTS

Patient	Age	Sex	Systolic Blood Pressure	Diastolic Blood Pressure	Hypertensin Found
			mm. of Hg.	mm. of Hg.	units per liter
V.D.D.	26	M	120	65	0.00
L.G.B.	64	M	120	80	0.00
J.G.	23	M	118	74	0.00
D.A.A.	20	M	130	80	0.00
M.F.B.	24	M	124	68	0.00
M.F.H.	32	M	114	60	0.00
R.R.R	26	M	114	90	0.00
J.A.	25	M	130	90	0.00
S.F S.	28	M	120	60	0.00
O.D.W.	26	M	130	74	0.00
R.C.P.	26	M	130	80	0.01
W.L.F.	37	M	120	70	0.02
J.G.B.	32	M	119	65	0.02
J.W.H.	53	M	115	80	0.02
W.T.W.	45	M	119	85	0.02
T.G.H.	33	M	116	80	0.02
G.H.	30	M	125	80	0.02
H.W.E.	27	M	135	84	0.02
P.J.L.	41	M	118	80	0.02
P.C.F.	41	M	125	75	0.02
J J.	36	M	126	79	0.03
S.W.	31	M	120	82	0.03
C.C.G.	29	M	124	70	0.05
F.B.	28	M	108	78	0.05

Table 28 illustrates the amount of hypertensin found in a group of 10 patients with malignant hypertension. The amounts found ranged between 0.08 and 0.43 units per liter. The average amount found in this group was 20 times greater than the average amount present in the normotensive patients. This is clearly a significant result and one can say that at least in malignant hypertension the rise in blood pressure is due to circulating hypertensin.

Table 29 shows the amounts of hypertensin found in a group of 18 patients with benign essential hypertension. The average for this group is 0.03 units per liter. When analyzed statistically the amount found in this group is significantly greater than the hypertensin found in the normotensive group.

TABLE 28

ASSAY OF HYPERTENSIN IN THE BLOOD OF PATIENTS WITH MALIGNANT ESSENTIAL HYPERTENSION

Patient	Age	Sex	Systolic Blood Pressure mm. of Hg.	Diastolic Blood Pressure mm. of Hg.	Hypertensin Found units per liter
C S	32	M	227	150	0.08
L.A.S	33	M	202	146	0.10
F J W.	32	M	205	149	0.11
R J.H	51	M	225	133	0.11
					0.31
A H A	62	M	220	120	0.24
J A G	38	M	206	134	0.24
G T	54	M	203	148	0 24
M J	48	F	300	160	0 40
L G	47	F	289	159	0.40
L T	42	F	247	149	0.43

TABLE 29

ASSAY OF HYPERTENSIN IN THE BLOOD OF PATIENTS WITH BENIGN ESSENTIAL HYPERTENSION

Patient	Age	Sex	Systolic Blood Pressure mm. of Hg	Diastolic Blood Pressure mm. of Hg.	Hypertensin Found units per liter
A.J.L.	36	M	204	135	0.00
A N.	56	M	173	111	0.00
A P	62	M	202	103	0.00
E E.A	59	M	187	112	0.00
A.J.L	36	M	204	135	0.02
F P T	55	M	210	142	0.02
C.A.	57	M	164	105	0.02
F A B	62	M	165	116	0 02
R L W	43	M	201	134	0.02
H P	55	M	198	130	0.03
A F B	44	M	146	100	0.03
M S	51	M	212	129	0.03
H E G	59	M	156	120	0 04
L A G.	63	M	195	115	0.04
F S	37	M	170	106	0.05
A F B.	44	M	146	100	0.05
C.D.K.	57	M	174	106	0.06
C M	54	M	206	125	0.06

TABLE 30

THE EFFECT OF INTRAVENOUS INJECTIONS OF HORSE HYPERTENSIN UPON THE BLOOD PRESSURE OF SEVERAL HUMAN SUBJECTS

Patient	Age	Sex	Dose	Control blood pressure	Maximum blood pressure
	yrs.		units	mm. Hg.	mm. Hg.
L.D.	41	M	2.5	120/82	190/120
				112/80	180/130
S.R.	44	M	2.0	120/90	174/140
				120/90	172/130
				128/90	180/140
D.S.	38	M	1.5	120/70	160/120
				110/92	165/120
M.J.	35	M	2.0	108/60	130/80
				110/50	130/80
				110/54	125/75
J.S.	46	M	2.5	110/72	160/100
				108/70	150/100
J.Y.	33	M	2.5	126/80	168/108

Table 30 illustrates the response of patients to the injection [24] of 1.5 to 2.5 Goldblatt units. The material used for injection was relatively pure having a specific activity of 5000 Goldblatt units per mg. of N. With this purity there were no side effects from the intravenous injection. In the second patient there was a rise in blood pressure of 54 mm. of Hg. in 1 minute. This is a large rise when one remembers that 2 Goldblatt units are diluted in the patient so that the maximum concentration of hypertensin which could possibly be attained in the blood would be 0.4 of a Goldblatt unit per liter We, therefore, feel that although the amount of hypertensin found in the benign phase is not striking, it is within the range of the amount that might be necessary for it to be the chemical mediator of benign essential hypertension, as well as in the malignant phase of the disease

Hypertensin, therefore, seemed to us an important physiological material and accordingly we embarked upon a program of purification.

[24] Skeggs, L. T., Marsh, W. H., Kahn, J. R., Shumway, N. P., J. Exper. Med. 99: 275, 1954.

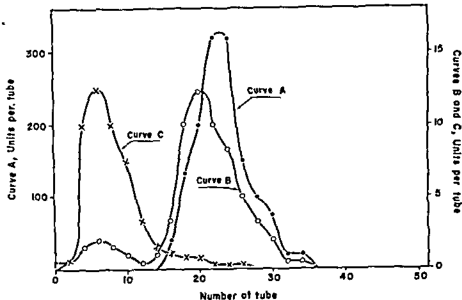


Fig 63 Results of counter-current distribution study on hog renin incubated with horse blood

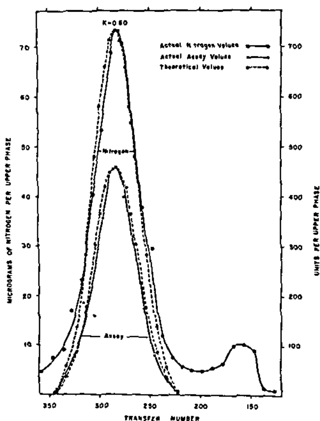


Fig 64. Analysis of material from counter-current distribution run

We soon learned that an excellent method of purification was by the countercurrent distribution method. When successive batches of impure hypertensin were subjected to this process, we found the active material in different portions of the countercurrent machine indicating the existence of two types of hypertensin [24]

When hog renin is incubated with substrate from horse blood in the absence of chloride and the resulting hypertensin subjected to a countercurrent distribution a result is obtained corresponding to curve A in Figure 63. If the peak tubes are removed from curve A, the material divided into two portions, and one portion reincubated with substrate in absence of chloride, material giving curve B is obtained. When the second portion is reincubated with substrate in the presence of chloride, curve C is obtained. This chemical transformation of one material to another is due to the action of a chloride activated enzyme present in the plasma This enzyme converts the material from curve A or B, called hypertensin I, to that material composing curve C, called hypertensin II.

A small study was carried out to determine what ions were able to activate the enzyme in addition to chloride. It was found as shown in Table 31 that chloride, bromide and nitrate would activate the enzyme whereas carbonate, phosphate and sulphate were ineffective.

Having found that there were two types of hypertensin, we choose to proceed with the purification of the first product of the action of renin on its substrate, designated hypertensin I

The purification [25] was accomplished by a series of steps illustrated in the flow diagram of Table 32

The crude hypertensin is obtained by incubation of the renin prepared from 400 lbs of hog kidneys with the substrate from 400 liters of horse plasma. The crude material thus obtained contains 120,000 units with a purity of 40 units per mg. of N_2. By use of an alumina column, by salting out, and by butanol extraction, the purity is increased to 4850 units per mg of N_2 At this point, the hypertensin is subjected to countercurrent distribution in a 106 tube machine using 375 transfers and the single withdrawal method of Craig [26] An analysis of one such run is presented in Figure 64. The material from the peak tubes, representing one component, was pooled and after concentration and precipitation was found to have a purity of 7050 Goldblatt units per mg. of N_2 or 1125 units per mg of solid. A portion of this material was hydrolyzed and chromatographed on paper in two dimensions using secondary butanol-NH_3 and phenol-H_2O solvents. The chromatogram shown in Figure 65 reveals the presence of 9 amino acids, which have been identified as aspartic acid, valine, proline, isoleucine, leucine, phenylalanine, tyrosine, arginine and histidine.

Hypertension I is truly a remarkably active pressor substance. On a weight for weight basis, it is 4 times stronger than l-arterenol which is one of the most powerful

[25] Skeggs, L T., Marsh, W. H., Kahn, J. R., Shumway, N. P., J. Exper. Med. 100: 363, 1954.

[26] Craig, L C., Methods Med. Research, 5: 3, 1952.

TABLE 31

THE EFFECT OF VARIOUS SALTS
UPON THE TYPE OF HYPERTENSIN FORMED

Experiment No.	Salt added	Predominant type of hypertensin
185	No salt	I
238	0.0001 m NaCl	I*
227	0.001 m NaCl	II*
208B	0.01 m NaCl	II
186	0.153 m NaCl	II
194A	0.1 m Na_2HPO_4	I
209B	0.1 m $NaHCO_3$	I
214A	0.1 m NaBr	II
214B	0.1 m NaF	II
204	0.1 m $NaNO_3$	II
194C	0.1 m KCl	II
212	0.1 m K_2SO_4	I
203	0.1 m $CaCl_2$	II
205A	0.1 m $MgCl_2$	II
205B	0.1 m $MgSO_4$	I
199A	0.1 m $(NH_4)_2SO_4$	I
199B	0.1 m NH_4Cl	II
194D	0.1 m Li_2SO_4	I

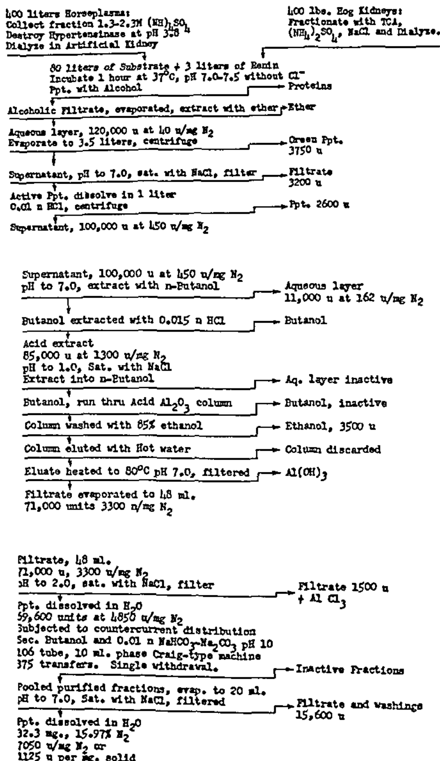
*These experiments yielded mixtures of the two types of hypertensin.

pressor materials Further study is necessary to finally establish the purity of this product.

CHAIRMAN METCOFF: Thank you, Dr Skeggs. Dr. Orbison will continue.

DR. JAMES ORBISON: My interest in this subject started from the standpoint of vascular disease, rather than from hypertension, and I will present another facet of this particular problem. Before I do that, however, I think it might be well to go into a little of the background that preceded this particular piece of work.

TABLE 32


PURIFICATION OF HYPERTENSIN I
400 liters Horseplasma:
Collect fraction 1.3-2.3M (NH)4SO4
Destroy Hypertensinase at pH 3.8
Dialyze in Artificial Kidney
400 lbs. Hog Kidneys:
Fractionate with TCA,
(NH4)2SO4, NaCl and Dialyze.
80 liters of Substrate + 3 liters of Renin
Incubate 1 hour at 37°C, pH 7.0-7.5 without Cl⁻
Ppt. with Alcohol
Proteins
Alcoholic Filtrate, evaporated, extract with ether
Ether
Aqueous layer, 120,000 u at 40 u/mg N2
Evaporate to 3.5 liters, centrifuge
Green Ppt.
3750 u
Supernatant, pH to 7.0, sat. with NaCl, filter
Filtrate
3200 u
Active Ppt. dissolve in 1 liter
0.01 n HCl, centrifuge
Ppt. 2600 u
Supernatant, 100,000 u at 450 u/mg N2
Supernatant, 100,000 u at 450 u/mg N2
pH to 7.0, extract with n-Butanol
Aqueous layer
11,000 u at 162 u/mg N2
Butanol extracted with 0.015 n HCl
Butanol
Acid extract
85,000 u at 1300 u/mg N2
pH to 1.0, Sat. with NaCl
Extract into n-Butanol
Aq. layer inactive
Butanol, run thru Acid Al2O3 column
Butanol, inactive
Column washed with 85% ethanol
Ethanol, 3500 u
Column eluted with Hot water
Column discarded
Eluate heated to 80°C pH 7.0, filtered
Al(OH)3
Filtrate evaporated to 48 ml.
71,000 units 3300 u/mg N2
Filtrate, 48 ml.
71,000 u, 3300 u/mg N2
pH to 2.0, sat. with NaCl, filter
Filtrate 1500 u
+ Al Cl3
Ppt. dissolved in H2O
59,600 units at 4850 u/mg N2
Subjected to countercurrent distribution
Sec. Butanol and 0.01 n NaHCO3-Na2CO3 pH 10
106 tube, 10 ml. phase Craig-type machine
375 transfers. Single withdrawal.
Inactive Fractions
Pooled purified fractions, evap. to 20 ml.
pH to 7.0, Sat. with NaCl, filtered
Filtrate and washings
15,600 u
Ppt. dissolved in H2O
32.3 mg., 15.97% N2
7050 u/mg N2 or
1125 u per mg. solid

Several years ago Dr. Grollman [27] postulated that the kidney had a function which *prevented the production of hypertension*. When the artificial kidney and peritoneal lavage became available, he found that keeping nephrectomized animals alive for a considerable number of days, ranging up to 79, resuted in hypertension in 28 of the 38 animals so treated. These results were believed to support the hypothesis of a renal antipressor effect.

Dr. Leonards and Dr. Heisler [28] thought that there might be some other explanation. They repeated the experiments following very closely the fluid and electrolyte balance in these animals. They found that the animals which became hypertensive were those which retained a significant proportion of the perfusing fluid.

With this, then, as a background, it seemed to me possible that rapid hydration *in bilaterally nephrectomized* dogs *might* be *expected* to *produce* hypertension and vascular disease in a relatively short period of time.

In a series of bilaterally nephrectomized dogs we found that only an occasional animal will develop hypertension.

Then we arbitrarily gave a large dose of isotonic sodium chloride solution (100 ml./Kg./day) to bilaterally nephrectomized dogs intraperitoneally. They developed hypertension in 24 to 48 hours, and at the time of death, 4 to 8 days later, had a significant amount of acute arterial necrosis.

Figure 65 shows gross lesions in the stomach, pancreas and heart of one of these dogs *In the stomach you can see that numerous mucosal hemorrhages have occurred* both in the pyloric and in the fundic region, but the fundus shows a larger proportion of hemorrhages. In the heart there are sub-endocardial hemorrhages, both in the left and in the right ventricle, and in some animals this extended to a considerable depth in the myocardium.

The pancreas in this particular animal is prominently involved by hemorrhage, and although I don't have *sections or slides to show it here, the small intestine* and colon were also involved in a similar process.

Figure 66 shows the changes which were evident upon microscopic examination. The *myocardium*, taken from a zone of interstitial hemorrhage, is characterized by *myocardial* necrosis and a zone of adjacent degenerated myocardium.

Figure 67 shows the arterial lesions in a section from the submucosa of the stomach. You see an artery which still retains a few of its nuclei, but although I don't

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[27] Grollman, A., Muirhead, E. E., and Vanatta, J.,Role of the Kidney in Pathogenesis of Hypertension as Determined by a Study of the Effects of *Bilateral Nephrectomy* and Other Procedures on the Blood Pressure of the Dog. Am. J. Physiol. 157: 21, 1949.

[28] *Leonards*, J. R. and Heisler, C. R., Blood Pressure of Bilaterally Nephrectomized Dogs. Federation Proc., 11 (No. 1, Pt. 1): 247, 1952.



Fig 65 Amino acid chromatogram of hypertensin



Fig 66 Gross lesions in stomach, pancreas and heart of bilaterally nephrectomized dog following intraperitoneal NaCl instillation

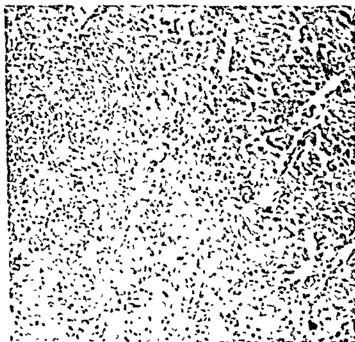


Fig 67a Myocardial necrosis and degeneration.

believe you can see it in this particular section, many of the nuclei in the wall are neutrophils. You also see the subintimal edema that raises the intima from the subintimal tissue.

Figure 68 is a section from the urinary bladder to show the typical fibrinoid change which has been described as a feature of malignant hypertension, both in the experimental animal and in the human. Hence, when these animals developed lesions, they were consistent with those seen in malignant hypertension. There was no question that these animals were overhydrated. They became obviously edematous.

✓Figure 69 illustrates the blood pressure changes in our "control" nephrectomized dogs. The blood pressures range from 110 to 140. We did our second nephrectomy on the eleventh day, and only one animal had a blood pressure as high as 160. One was 150 at the time of death The remaining animals were in the 130 or below range.✓

✓This is about the distribution of blood pressure that has been reported in the past for this type of experiment. Occasional animals do develop hypertension, but by far the greater number do not.✓

✓In contrast to this, when we gave bilaterally nephrectomized animals sodium chloride intraperitoneally, all with one exception had, within 24 to 48 hours, a blood pressure of 150 mm. Hg. or over, some ranging as high as 195. The one animal that did not get up to 150 previously had a very low blood pressure, and this rose some 30 mm. Hg. (Fig. 70.)✓

✓When we repeated the experiment giving modified Locke solution [29] instead of the sodium chloride, we found to our surprise that the blood pressure elevation was even more rapid, and that the degree of elevation was greater than that obtained with isotonic sodium chloride (Fig. 71.)✓

It was intriguing to find that the animals given a solution which was more nearly physiologic than sodium chloride should react by developing a greater degree of hypertension than the animals given the sodium chloride.

Because of this observation, we have used several other kinds of fluid, taking the modified Locke solution as a model, but varying it by making it up with chloride as the only anion or sodium as the only cation. In none of these instances have we found a solution which has a pressor effect as great as that of the modified Locke solution

Now we return to the distribution of lesions as they occurred in these animals When the nephrectomized dogs are given no fluid, lesions occurred in only two animals, (Table 33) This chart was made by assessing the lesions in the stomach, heart, and colon, from 1 to 3+, adding these together and then taking the average value. In the dogs given no fluid, there was only a plus-minus lesion in one dog and a 1+ lesion in another In dogs given normal saline intraperitoneally, two developed no lesions, the

[29] Skeggs, L T., Jr. and Leonards, J. R., Studies on the Artificial Kidney. I. Preliminary Results with a New Type of Continuous Dialyzer, Science 108. 212, 1948.

rest developed lesions, but of mild degree. In comparison, the dogs given the balanced salt solution or modified Locke solution developed very severe lesions in all of the organs examined.

TABLE 33

Nephrectomized Dogs Given No Fluid	Dog No.	27	34	35	36	37	39	41
	Degree of Vascular Lesions	-	-	-	+	-	-	±
Nephrectomized Dogs Given Normal Saline IP	Dog No.	28	29	30	31	32	38	40
	Degree of Vascular Lesions	-	++	++	+	+	-	++
Nephrectomized Dogs Given Salt Solution IP	Dog No.	43	44	45	46	47	50	53
	Degree of Vascular Lesions	+++	+++	+++	+++	+++	+++	+++

So here again we have some evidence which suggests that this may not be only a fluid factor, but that the type of electrolyte used may also play a role.

Since there is the possibility that the increased blood pressure was a direct result of an increase in blood volume in these edematous, over-hydrated dogs, it became necessary to study fluid volumes. T-1824 was used for determining blood volume, and inulin for determining extracellular fluid volume.

These methods have definite limitations, but we ran a control determination as soon as the second nephrectomy was completed, and then repeat determinations during the life of the animal. The results show that there are very definite increases in inulin space and variable changes in the blood volume.

The "control" dogs had changes in blood volume ranging from minus 8% to plus 12%. In our experimental dogs with sodium chloride solution, the changes ranged from a minus 15% to a plus 56%; three of these animals ranging from 5 to 15%. (Fig. 72.) This illustration also shows a group of dogs given the modified Locke solution. The blood volume range changes from minus 15 through a plus 75. In all dogs which we have done subsequently, we have found this peculiar inconstant change in blood volume, some dogs showing little or no change even on repeated determinations. Hence, I do not believe this is an experimental error.

There seems, therefore, to be an individual factor in the dogs which plays some role in control of blood volume and the hypertension. Hence, the hypertension is not simply a result of increased blood volume.



Fig 67b. Arterial lesion in submucosa of stomach

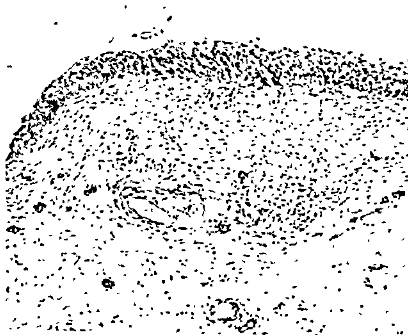


Fig 68. Fibrinoid change in urinary bladder of hypertensive overhydrated dog

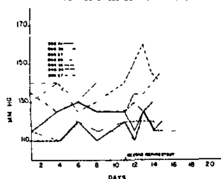


Fig. 69

Fig 69 Blood pressure levels in "control" nephrectomized dogs

Fig 70. Blood pressure levels in nephrectomized dogs after intraperitoneal instillation of NaCl

Fig. 71. Blood pressure levels in nephrectomized dogs, but after instillation of Locke's solution instead of NaCl

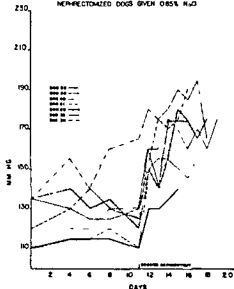


Fig. 70

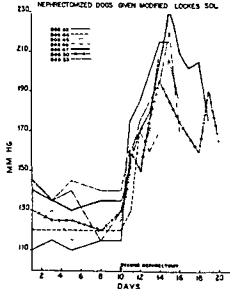


Fig. 71

In contrast, Figure 73 demonstrates the constant increase of interstitial fluid. It should be noted that in control animals which had no fluid over a period of 4 to 5 days, there was an increase of up to 40% in extracellular fluid. The experimental animals, as one would anticipate in this type of fluid overloading, had a marked increase in extracellular fluid, ranging from 200% to 400%.

As a result of these studies, we concluded that the increase in blood pressure was not the result of an increase in extracellular fluid Furthermore, the absence of the kidneys excluded the possibility of the hypertension being due to the presence of a renal pressor substance And finally, the fact that bilaterally nephrectomized dogs did not develop hypertension until they were overhydrated indicated that this hypertension was not due to the loss of a renal anti-pressor substance.

Work done by Kolff and Page [30], using this technique, adds a very interesting aspect to this problem. They have perfused kidneys from normal dogs with the blood of these hypertensive, overhydrated animals. When that is done, the blood pressure in the majority of the experimental nephrectomized animals drops to normal in approximately 2 hours. One immediately thinks. Is this then the result of overcoming the dehydration?

I think the answer to that is definitely "no," because in 2 hours the amount of fluid excreted by these donor kidneys is much less than would be necessary to alter hydration It has also been found that perfusing the hind legs of animals has produced no change in blood pressure, even though the amount of fluid as edema that was picked up by the donor leg, in some instances was as great as the fluid excreted by the kidney.

Obviously, then, we have the kidney back in this picture, because the evidence suggests that the removal of the kidneys has altered the body in some way which allows hypertension and arterial disease to occur when the animals are overhydrated.

CHAIRMAN METCOFF: Thank you, Dr. Orbison. We will proceed to Dr. Bumpus and Dr Page and then following this have a brief question period, before moving on to discussion of Salt Retaining Substances. Dr. Bumpus.

DR BUMPUS. In our laboratory at the Cleveland Clinic we are attempting to purify angiotonin However, our approach has been slightly different from the one reported earlier by Dr Skeggs

Our angiotonin preparations were assayed on a specially prepared dog whose sensitivity to angiotonin has been increased 5 to 10 fold over that of a normal animal. The unit of angiotonin activity is approximately 1/5th of the Goldblatt unit as described by Dr Skeggs.

It was felt that to obtain a purer angiotonin we should first purify renin substrate. This work was carried out in our laboratory by Dr. Arda Green and myself.

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[30] Kolff, W T. and Page, I. H., Blood Pressure Reducing Function of the Kidney; Reduction of Renoprival Hypertension by Kidney Perfusion. Am. J. Physiol. 175: 75, 1954.

The purification of renin substrate was carried out in 4 steps. Each step included a series of precipitations or extraction.

Series I is the usual preparation of renin substrate originally described by Plentl, Page and Davis. It consists of an ammonium sulfate fractionation at approximately pH 6, and collecting the precipitate between 1.64 and 2.1 M-ammonium sulfate concentration. This is repeated until the precipitate has a blue-green hue.

In Series II use is made of preferential denaturation of proteins. This has been utilized before to denature enzymes which hydrolyze angiotonin. However, we denatured many additional blood proteins and finally removed them by ammonium sulfate fractionation By the above-described procedure a purification of approximately 10 fold was accomplished.

Series III is another ammonium sulfate fractionation at about PpH 6.

In Series IV the substrate was adjusted to pH 2.5 and fractions were collected at three concentrations of ammonium sulfate.

With these four series of fractionations, we were able to purify renin substrate from 1000 to 1500 times that in blood serum.

Obviously, this preparation is not pure renin substrate. As shown electrophoretically, it contains both alpha and gamma globulins It is interesting to note here that Plentl, Page and Davis [31] reported a renin substrate preparation which contained only alpha globulin, yet it had a much lower specific activity than does the one that we *are reporting here* ✓*From this one can easily infer that renin substrate is an extremely* small fraction of the serum alpha globulins.✓

It became evident that further purification of renin substrate after series III gave no further purification of the angiotonin formed after incubation with renin. However, the product from series III substrate was about 50 times more pure than the product from series I and the yield of angiotonin increased about 3-fold.

The procedure for the purification is much simplified compared to most that have been reported. There is nothing unusual in the steps as shown. The incubation mixture was first deproteinized with ethyl alcohol. After evaporation to about 1/10th the original volume, the angiotonin preparation was freed of fatty materials and some protein was removed by allowing it to stand at pH 1.8 and at -20°C. Numerous fractions were then obtained by the serial addition of sodium cloride at pH 1. The recovery from this fractionation was good, usually being about 95%. More than 50% of the angiotonin recovered from this step was pure enough to be partitioned in a counter-current machine.

For the purification of angiotonin by partitioning we chose a solvent system of 1:1 butanol-propanol equilibrated against 0.1 N hydrochloric acid. This system having

[31] Plentl, A. A., Page, I. H., and Davis, W. W., J. Biol. Chem. 147: 143, 1943.

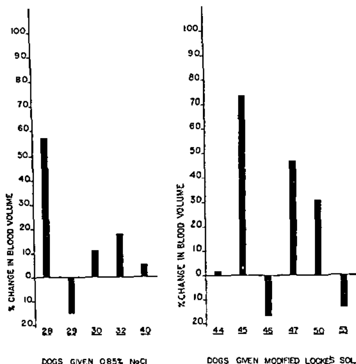


Fig 72 Blood volume changes in nephrectomized dogs

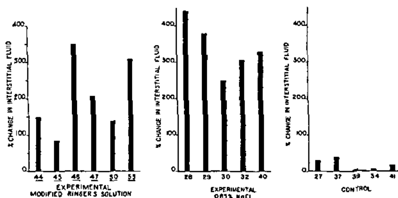


Fig. 73 Interstitial fluid volume changes in nephrectomized over-hydrated dogs

a pH of about 1.2 was used because it gave (1) a usable distribution of angiotonin, (2) it showed no destruction of the activity and (3) the final product, after lyophilizing, was essentially salt-free.

LARGE SCALE PREPARATION OF ANGIOTONIN

RENIN + RENIN SUBSTRATE ⟶ ANGIOTONIN

1. Deproteinize by addition of ethyl alcohol
2. Clarify by allowing to stand at pH 1 8 and at -20°C.
3. Fractionate with NaCl at pH 1.0
4 Partition between butanol-propanol: 0.1 N HCl

On Figure 74 is shown an activity curve of angiotonin run 100 transfers in the above mentioned solvent system The theoretical curve, the dotted line, fits quite closely to the actual curve. This comparison is not much of an indication of purity, it shows only that there is but one pressor substance present in this preparation Upon removal of the fractions from the center of the activity peak after counter-current distribution and redistributing in the same solvent system for 120 transfers, we were able to increase the purity but slightly However, the angiotonin samples at the top of the activity peak were always purer than those on the sides.

In order to determine purity, we resorted to a chemical method. Samples were taken from both sides of the activity peak near the top and the amino end group was determined by the Sanger method In each case, only one amino acid was found, namely, aspartic acid. From this we felt that even though the angiotonin was not pure, it was close enough to purity to justify some work on its structure.

In Figure 75 is shown a two dimensional chromatogram of an acid hydrolyzate of angiotonin. We show more amino acids here than reported earlier today by Dr Skeggs. However, our hydrolyzate lacks tyrosine while that of Skeggs and Kahn contains tyrosine It is interesting to note that these two preparations of angiotonin have almost identical specific activities, (our preparations having a specific activity of 30,000 to 35,000 units per milligram of nitrogen), but there is species difference. Our renin substrate was made from hog blood while that of Skeggs and Kahn was made from horse blood.

Our proposed structure of angiotonin is shown below. The amino acids in the angiotonin hydrolyzate were determined quantitatively on a Dowex 50 column and their ratios are shown here. As was stated earlier the amino end group was found to be aspartic acid

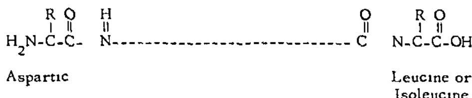
By using the hydralzinolysis method we were able to determine that either leucine or isoleucine was the terminal amino acid at the carboxyl end of the peptide. The remaining amino acids, after subtracting the two for the end groups go to make up the center of a straight chain peptide

We have proceeded further and isolated fragments of this peptide and identified four of the amino acids from the amino end fragment.

At this time, however, we were able to show by several means that our preparations were not as pure as we had originally thought; so consequently, our work on the structure is not valid. So now our work has turned back to further purification of angiotonin.

AMINO ACIDS IN ANGIOTONIN

2 Aspartic	1 Alanine	2 Leucine
1 Serine	1 Valine	2 Histidine
1 Glutamic	1 Isoleucine	1 Lysine
2 Proline	1 Tyrosine	2 Arginine
1 Glycine	1 Phenylalanine	



CHAIRMAN METCOFF· Thank you, Dr. Bumpus. Dr. Page, would you like to continue?

DR. IRVINE PAGE: Mr. Chairman, Ladies and Gentlemen: I gather that I am in the cleanup spot. You have heard the recitation of a large amount of, I think, very beautiful work.

I am sure Dr. Goldblatt would share with me the view that we would like to turn back to the old days when hypertension was all very simple, and we had it in the bag.

Now it turns out to be expremely complicated; these complications, I am sure, you have recognized.

I was slightly put to it to know what to say, not to duplicate the many things said earlier, so I am taking as my thesis: Why the extraordinary spontaneous change in the cardiovascular response of various animals, and human beings, to standard stimulations?

Just to orient you, Figure 76 shows the various equilibrated components of the system controlling the perfusion of tissues.

What are all these various factors? Well, you know there are people who believe in chemical mechanisms, in neural mechanisms, change in elasticity, cardiac output,

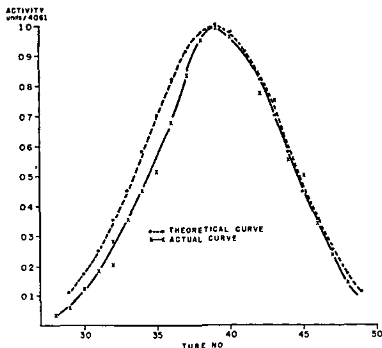


Fig 74. Countercurrent distribution of angiotonin using Butanol-Propanol: 0 1 N HCl

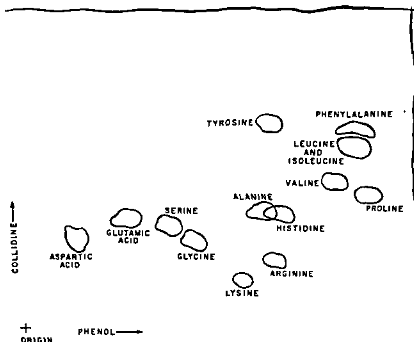


Fig. 75 Two dimensional chromatogram of acid hydrolysate of angiotonin

vascular caliber, viscosity, volume, and this one, reactivity, which I am going to discuss a little more in detail today.

The responses to angiotonin and another pressor agent which is produced by the action of pepsin which is known as pepsitensin are shown in Figure 77. You don't hear much about the latter these days.

I suspect as the angiotonin problem is better clarified, pepsitensin will come into its own. They are closely related, although not identical substances.

We always assume that an animal should respond to drugs in what we assume to be a "normal" fashion; the whole thing hinges on the term "normality."

You know perfectly well that one patient shows one type of response and another shows a very much lesser or greater response to a standard dose of a drug.

This intrigued us because we were concerned with the problem of how to increase the sensitivity of assay animals for angiotonin. When it was in such small supply any increase in cardiovascular reactivity in the test animal made a vast difference.

I won't trouble you with all the various things that we have found which almost specifically influence the response of the animal to a wide variety of vaso-active drugs. Let me call your attention to just a few of them: Destruction of the spinal cord, section of the spinal cord at C_6, lumbo-dorsal sympathectomy, autonomic ganglion blockade, all influence the ability of the animal to respond to vaso-active materials. Hepatectomy is one of the best ways to reduce cardiovascular reactivity. Eight to 10 hours after the removal of the liver, these animals become refractory to almost all vaso-active drugs.

One wonders perhaps if the death of the animal after hepatectomy might not, in large measure, be due to this development of refractoriness. The same sort of thing occurs in hemorrhagic and traumatic shock, as you probably know. There are a variety of mechanisms which control, I think, in an equilibrated fashion the ability of the body to respond to stimulating or depressing agents. These are all included in what we call "cardiovascular reactivity."

Here are some examples of changes in reactivity.

Figure 78 shows response to injections of renin, and as you see, they gradually diminish. At point (9) the autonomic ganglion blocking agent tetraethylammonium chloride was given and immediately the response to renin returns. The blockade of autonomic ganglia caused this remarkable change in the ability of the animal to respond.

Figure 79 shows response to angiotonin (1 and 4). You can change the animal's responsiveness remarkably.

Since angiotonin is the thing we are primarily interested in this morning, the question is: Is there any degree of specificity involved in these reactivity changes?

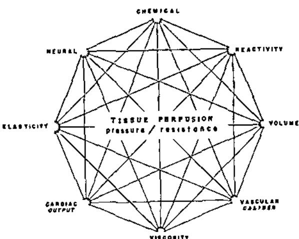


Fig 76 Components controlling perfusion of tissues by blood

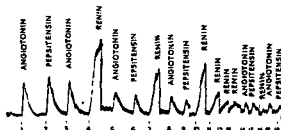


Fig 77 Effect of pepsitensin and other pressor agents on blood pressure

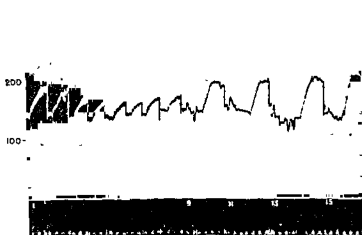


Fig 78 Effect of tetraethylammonium chloride (TEAC) on waning response to repeated renin injections

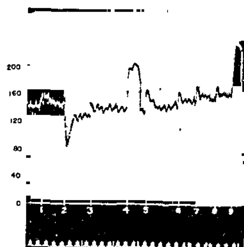


Fig 79 Response to angiotonin

It is conceivable that a person with abnormal specific sensitivity to angiotonin might well develop hypertension. If the sensitivity were great enough even a normal amount of inoculating angiotonin could produce hypertension.

This is an example in animals to show you that there are certain peculiar aspects to this increased responsiveness to angiotonin.

Figure 80 illustrates response to an injection of noradrenaline. Tetraethylammonium chloride was given in order to augment the response, and then the carotid sinuses were sectioned. At this point, one still gets a marked rise in arterial pressure even though one might think the autonomic ganglia were blocked.

Figure 81 shows the opposite effect.

Here tetraethylammonium chloride was given, then renin, and serotonin. Carotid sinuses were sectioned in the presence of the tetraethylammonium chloride, and now the injection of renin is greatly augmented but that of serotonin is not.

Figure 82 shows the same thing with angiotonin.

Injection of tetraethylammonium augmented noradrenaline but there is some further augmentation with noradrenaline and with angiotonin The carotid sinuses were sectioned, and then the angiotonin effect is greatly augmented, whereas, the noradrenaline is not augmented further.

I really don't know whether these relatively specific changes are of importance in the etiology of hypertension.

Subcutaneous injection of renin ordinarily gives no rise in blood pressure. When these animals are sensitized, the injection of renin gave a very prolonged rise which lasted for as long as it was recorded, 11 hours. (Fig 83) The possibility exists that change in substrate sensitivity would make it possible for extremely small amounts of vaso-active agents to give very prolonged rises in arterial pressure.

I would like to close by saying that I have only made the problem a little more complicated; my hat is off to the people preceding me who showed you the gradual unfolding of the very complicated renal-vasopressor system. There was a day, which I ought well to remember, when it too seemed hopelessly complicated It has now been fifteen years since we discovered that renin is an enzyme which acts on a substrate to produce the peptide angiotonin. In the interval many workers have joined in trying to clarify the position of this complicated renal-vasopressor system among the mechanisms of hypertension. Some think all hypertension is produced by it, others see no evidence for its participation at all. I have always believed it participated in some, but only to a minor degree, if at all, in others. The rigid proof must await further knowledge of the chemistry of angiotonin. Even today it is quite apparent that its activity as a pressor substance exceeds that of other pressor agents, which means it will probably be present in blood in very minute amounts. Some of you have asked me about terminology. We use the name which we gave the substance when we discovered it,

avoiding implication as to whether it was or was not the hypertensive agent. The South American group under Braun-Menendez arrived at about the same conclusions we did, at about the same time. They called their product "hypertensin."

Whether you prefer the home-grown product, "angiotonin," or the more exotic one from the pampas, "hypertensin," is a matter of choice. Less satisfactory is the term "hypertensinogen" for renin-substrate. It is misleading as regards the type of chemical reaction occurring, indeed, suggesting that of trypsinogen and pepsinogen.

Ultimately the various panels of my octagon will be investigated by the bright youngsters of the future and I have no doubt it will eventually be fitted together into a beautifully integrated, equilibrated system controlling perfusion of tissues.

CHAIRMAN METCOFF: This has been a magnificent opportunity for many of us to become acquainted with a field in which we ordinarily have little experience, and the complicated material has been presented in a most lucid way.

I imagine there are going to be many questions.

First of all, a question for Dr. Skeggs: If it is possible to isolate the hypertensive factor by dialysis, does dialysis reduce the hypertension in the patient with hypertension?

DR. SKEGGS: No, the amount that passes through the cellophane is a small portion of the circulating hypertensin and, therefore, there is no decrease in the animals' blood pressure.

DR GOLDBLATT: I would like to ask Dr. Orbison whether he practices removal of the fluid he injects intraperitoneally or whether he leaves it in.

DR. ORBISON: The fluid is injected every day. This is not a peritoneal lavage, but a daily addition of fluid to the animal.

DR. GOLDBLATT· You mentioned azotemia. Do they all have it?

DR. ORBISON· All of these dogs show azotemia. The animals that are hydrated show a slightly lesser degree of azotemia, but I believe that is only due to the fact that the nitrogenous products are spread through a greater volume of fluid.

DR. CONRAD RILEY: Did you try injections of non-electrolyte fluid? I suppose it dilutes the animals up so fast that it kills them rapidly.

DR ORBISON: We attempted that. As you anticipated, if you disturb the electrolyte balance so greatly, the animals die in a short period of time.

We have cut the injected sodium in half, and we do get elevation in blood pressure, but it is less, and there is less vascular disease than if we use more nearly isotonic solution.

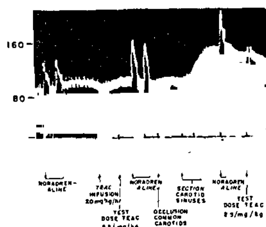


Fig 80 Effects of TEAC and carotid sinus section on noradrenaline responsiveness

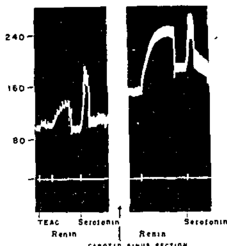


Fig 81 Effect of TEAC and carotid sinus section on response to renin and serotonin.

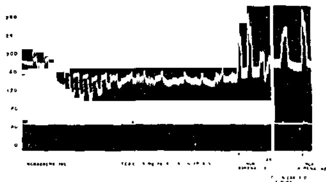


Fig. 82. Effects of TEAC and carotid sinus section on response to noradrenaline and angiotonin.

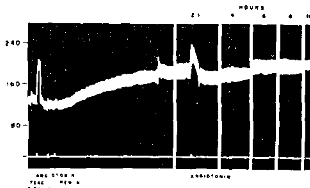


Fig. 83 Effect of subcutaneous injection of renin after animal sensitized to renin.

DR. LANGE: How about plasma?

DR. ORBISON: No, we have not used plasma.

DR. BARNETT: What is the composition of the modified Locke solution?

DR. ORBISON: Sodium 148 meq/L; potassium, 3; calcium, 5; and magnesium, 3 meq/L. Then the anions, chloride, 125, and the rest are a mixture of phosphate, carbonate and lactate.

DR. MILLER: Dr Orbison, the artificial kidney can be used for removal of electrolytes and fluid. Have you tried reversing hypertension and the lesions by that means? In other words, after you have given the excessive fluid and electrolyte and hypertension developed, can you maintain the animal by dialysis?

DR ORBISON. I have not attempted that. Perhaps the work that Dr. Kolff and Dr. Leonards have done approaches that. I am not sure it does exactly. Dr. Kolff used the perfusion of kidneys after animals had supposedly developed lesions, or would be expected to develop lesions, and were certainly hypertensive and reversed the hypertension very quickly. Lesions were present in some of those animals at the time of autopsy.

Dr Leonards found that in some of his animals he could regulate the fluid volume by using hyper and hypotonic solutions in either his peritoneal lavage fluid or artificial kidney, when he used hypertonic solutions to perfuse and draw out more fluid, the blood pressure dropped, and as he replaced fluid with hypotonic solutions, the blood pressure rose.

DR MILLER· There is a difference between the experiments Kolff has done where the normal kidney is perfused because presumably the hypertensive substance could be removed, whereas, the artificial kidney does not remove sufficient amounts of hypertensive substance but could remove the electrolyte, and thus possibly might clarify the problem

DR ORBISON: There is a time difference between the two sets of experiments. That is probably important. When the kidney is used, the change in blood pressure occurs in a very short period of time, a matter of hours, without a concomitant change in fluid, whereas, in Leonards' experiments, the change in blood pressure followed the change in fluid and was a matter of days

DR. JANEWAY I am very ignorant in this field and probably somebody can set me straight very quickly, but I had an idea that another variable was the adrenal which in some way is necessary to maintain blood pressure, and which it would seem to me in such experiments as Dr. Orbison described would probably be put under a tremendous strain and be doing all sorts of things which might have terrific effect on the tissues when there was no end organ in terms of renal tubules to work on.

Where does the adrenal cortex relate to all that has been described today?

DR. GOLDBLATT: It is certainly a definite fact that the only thing that has ever interfered with the elevation of blood pressure on the basis of constriction of the main renal arteries has been the removal of both adrenals. In the earliest experiments performed, we destroyed the medulla of one adrenal and excised the other adrenal. When the renal arteries were then constricted the hypertension still developed. But if both adrenals were ablated, which was the only way to remove the cortex of both adrenals, there was no rise of blood pressure when the renal arteries were constricted. We concluded that an adequate amount of adrenal cortex must be present to permit the elevation of blood pressure to occur as a result of constriction of the renal arteries. We have investigated this phenomenon, but I shall not go into the details on this occasion.

In years gone by, whenever I came to this part of the subject, I used to say, "Don't believe for one moment that I am advocating the removal of both adrenals for the treatment of hypertension." As you know now, however, that day has come, and that procedure is actually being practised on human hypertensives.

DR. BARNETT: Hypophysectomy does not interfere with the development of hypertension?

DR. GOLDBLATT: That is right.

DR. GEORGE SAYERS: Isn't it true, Dr. Goldblatt, that all one needs is a replacement dose of adrenal cortical substance to maintain the hypertensive state in these animals?

DR. GOLDBLATT: Yes.

DR. SAYERS: I think that is an important point in considerations of the etiological role of the adrenal cortex in hypertension.

It brings up the matter of two philosophies as to the role of the adrenal cortex in disease, one being the general adaptation syndrome that thinks of hyperactivity of the adrenal cortex as being the etiological factor in the development of hypertension, and the other philosophy which has been posed and backed up by the beautiful experimental data of Dingle, that indicates the adrenal cortex plays what he calls a permissive role. In other words, according to this permissive theory, there has to be a little of the cortical hormone around for the smooth muscle of the cardio-vascular system to manifest itself.

It effects the dose in the hypertensive state so that maintenance of the hypertension doesn't require an excessive secretion of the adrenal cortex, simply requires the normal output.

DR. GOLDBLATT: When we reduced the cortical substance in a few animals to about 1/5th or 2/5ths of the original amount, and then constricted the renal arteries, up went the blood pressure in the usual way.

DR PAGE: I think you might add that there is a further complication; even with total adrenalectomy in some patients, the blood pressure does not always come down. It is a fly in the ointment we would like to overlook because certainly all evidence from a variety of sources, as Dr. Goldblatt has said, is in favor of the fact that removal of adrenal cortices causes a fall in blood pressure to normal, and it can be brought back to the hypertensive level by a variety of adrenal steroids. It comes somewhat as a shock in patients to find that -- and even to surgeons it comes as a shock -- removal of both adrenals doesn't always lower the pressure to normal. This is a disturbing phenomenon

DR GOLDBLATT: It should not be surprising because substitution therapy is being practised in such cases

DR. PAGE: The trouble is, when we dropped the adrenal cortical support from the patient, the blood pressure did not come down. One patient had two bouts of severe adrenal insufficiency with marked rise in serum potassium and fall in sodium while receiving steroid therapy without significant fall in arterial pressure. In malignant hypertension, hypertension can be maintained in the face of adrenal insufficiency.

DR. ARTHUR MERRILL Will it come down with treatment, etc.?

DR. PAGE: Blockage with hexamethonium, as you know, knocks the spots out of patients temporarily, and many of them become refractory We have found some of the hypertensives do not show a fall with even very large amounts, 200 milligrams, of hemethonium intravenously. It is this variability of response whether to adrenalectomy, hexamethonium, reserpine, sympathectomy, or what you will, that makes me think hypertension in patients is either of multiple etiology or a multi-faceted disease with predominance of one facet over the others in varying proportions.

DR GOLDBLATT: I would like to ask Dr Skeggs whether he has tried to determine whether a given dose of hypertensin will cause an increased pressor response in the patient with hypertension over that produced in the normal.

DR SKEGGS: No

CHAIRMAN METCOFF: We are indebted to each of the participants for a stimulating session Since time is limited, let us proceed to a discussion of salt retaining hormones

V. SALT RETAINING ADRENAL CORTICAL STEROIDS

CHAIRMAN METCOFF: We will begin with Dr. Luetscher and a discussion of Salt Retaining Substances in the Urine. Dr. Luetscher.

DR. JOHN A. LUETSCHER: It has been known for a number of years that the amorphous fraction of adrenal cortical extract contains sodium-retaining activity beyond that accounted for by its content of known, crystalline corticosteroids.

The situation remained static for a period of almost twenty years until a more sensitive bio-assay of sodium-retaining hormone and the use of paper chromatography for the fractionation of adrenal cortical steroids allowed the reopening of the subject.

A number of investigators on both sides of the Atlantic reinvestigated this problem, and groups in this country and England have prepared a crystalline corticosteroid of very high sodium-retaining activity. Subsequently, collaboration of the British and Swiss groups led to its identification as the 18-aldehyde derivative of corticosterone, or aldosterone [1-4].

This material exists in body fluids as the hemi-acetal, bridging the 11 and 18 carbons. It is responsible for the bulk of the sodium-retaining activity of adrenal cortical extract, of adrenal venous blood, as Dr. Farrell will show us, and of the urine.

The same developments which made this work possible also came to our attention beginning in 1948, and our investigations were aimed at the demonstration of a sodium-retaining material. Our reason was that we were dissatisfied with the concept that glomerular filtration rate regulated sodium excretion. In patients with nephrosis, during the injection of concentrated human serum albumin, the filtration rate could be raised from subnormal to supranormal levels, doubled in some instances, with small and sluggish changes in sodium excretion [5]. These studies convinced us that the tubule had the last word in sodium excretion, and that there must be something to account for

[1] Mattox, Mason, and Albert, J. Am. Chem. Soc. 75: 4869, 1953.
[2] Knauff, Nielson, and Haines, J. Am. Chem. Soc. 75: 4868, 1953.
[3] Simpson, Tait, Wettstein, Neher, v. Euw, Schindler, and Reichstein, Experientia 10: 132, 1953.
[4] Harman, Ham, de Young, Brink, and Sarett, J. Am. Chem. Soc. 76: 5035, 1954.
[5] Luetscher, Hall, and Kremer, J. Clin. Invest. 29: 896, 1950.

increased tubular reabsorption of sodium in the nephrotic patient.

When Addis [6] pointed out that experimental proteinuria also seemed to be dependent on the presence of adrenal corticosteroids, it was easy to speculate that in the nephrotic syndrome, in addition to the obvious renal lesion, there was some superimposed factor, originating in the adrenal cortex, which was affecting the excretion of sodium and perhaps also that of protein.

We then set up a method for bio-assay of sodium-retaining activity [7,8]. Dr. Quentin Deming was responsible for many of the early observations, and since that time, Drs. Edward Hyman, Ben Johnson, Joseph Cates, and Bernard Axelrad have been working with the group They are responsible for most of the data which I am going to show today

In Figure 84 we see plotted on the vertical parameter the sodium-retaining activity of urine extracts, expressed as micrograms of DCA equivalent per 20 minutes of urine, that is, 1/72 of a 24-hour specimen On the bottom line, plotted on a log scale, is total urine sodium per day As you see, the sodium output shows a good inverse correlation with the output of the sodium-retaining corticoid.

Increased sodium-retaining activity was found not only in the nephrotic syndrome, but also in other diseases associated with edema. The X's are children with the nephrotic syndrome. Solid circles and squares are adult patients with nephrosis. The triangle is a patient with cirrhosis of the liver. Patients with cardiac failure are indicated by dots within circles and squares. Sodium-retaining activity of a smaller degree was found in normals on low sodium diets In all of these groups, shown on the chart, we see that there is a general relationship. The higher the sodium-retaining activity of the urine extract, the lower the urine sodium [9].

CHAIRMAN METCOFF Does negative sodium-retaining activity mean sodium-excreting activity?

DR LUETSCHER. Yes. This, in a sense, is an artifact of the bio-assay. It has been useful to us in our work on fractionation because substances like cortisone, hydrocortisone and corticosterone produced an increased output of sodium under the conditions of bio-assay The only natural compounds which are known to be sodium-retaining are aldosterone, desoxycorticosterone, and Reichstein's Substance S The order of activity of these materials is different by a factor of several hundred. Aldosterone is approximately 30 times as active as DCA, while Reichstein's Substance S is only a few per cent as active as DCA

When an actively sodium-retaining urine extract is chromatographed on paper in toluene and propylene glycol, the sodium-retaining activity is concentrated in a fraction

[6] Addis, Marmorston, Goodman, Sellers, and Smith, Proc. Soc. Exper. Biol. and Med 74. 43, 1950
[7] Deming and Luetscher, Proc. Soc. Exper. Biol. and Med. 73. 171, 1950.
[8] Johnson, Endocrinol. 54: 196, 1954.
[9] Luetscher and Johnson, J. Clin. Invest. 33: 1441, 1954.

which has approximately the same mobility as cortisone. This fraction reduces neo-tetrazolium and shows U-V absorption with a peak near 240 mμ. [10] With different chromatographic systems described by Bush, we have been able to prepare a substance with properties identical with those of aldosterone in biological activity and in the chromatographic behavior of the native material, of its monoacetate and diacetate, and of the oxidation products after treatment with potassium periodate [11].

Samples of this material have been submitted to Drs. Wettstein and Neher of the Ciba Research Institule at Basle. The crystalline form, melting point, and infrared spectrum of the sodium-retaining corticoid are identical with those of authentic samples of aldosterone [12].

It seems quite clear that the sodium-retaining corticoid, found in the nephrotic syndrome, is aldosterone We have assumed that the material of similar chromatographic mobility, with similar biological activity, found in various other conditions, is probably aldosterone also

We have been working entirely on urine. It would be desirable to measure aldosterone in blood. Unfortunately, the bio-assay is scarcely sensitive enough to determine normal levels in human blood, unless very large samples are used [13]. Furthermore, the blood level reflects only the situation at a given moment, while the output of material in the 24-hour urine has proved to be a satisfactory method for the assessment of the other secretions of the adrenal cortex

When normal urine is extracted in the neutral state, very little or no activity is found. When this urine is acidified to pH 1 and promptly extracted, perhaps a little activity is found, but in many instances the extract is diuretic. When this urine is allowed to stand at pH 1, at room temperature for 24 hours, considerably more material can be extracted, and an assayable quantity is obtained from normal men on ordinary diets.

When the normal individual goes on a low-sodium diet, aldosterone is found in increased amounts in extracts prepared after hydrolysis with acid or with beta-glucuronidate [14].

Many of our patients are on low sodium diets, and obviously we have to take this into account in the interpretation of the clinical data which we will show.

The excretion of aldosterone in the patient with nephrotic syndrome or heart failure, who is in a sodium-retaining phase of the disease, is larger than that in normal individuals, even those who have been on stringent sodium restriction. In certain instances as much as 20 times the normal output of aldosterone has been garnered in all of these fractions. In nephrosis and in heart failure, aldosterone is not extracted in

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[10] Luetscher and Johnson, J. Clin. Invest. 33: 276, 1954.
[11] Luetscher, Recent Progress in *Hormone Research*; in press.
[12] Luetscher, Neher, and Wettstein, Experientia 11: 456, 1954.
[13] Simpson and Tait, Recent Progress in Hormone Research; in press, 1955.
[14] Axelrad, Cates, Johnson, and Luetscher, Brit. Med. J.; in press, 1954.

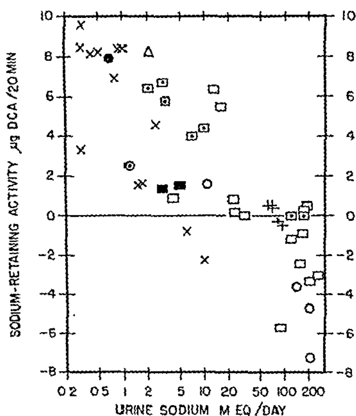


Fig 84 Relation between sodium-retaining activity of urine and daily output of sodium.

significant amounts from neutral urine, but is easily extractable from acidified urine. After standing at pH 1, or after treatment with glucuronidase, still more aldosterone is recovered.

It is apparent then that the method of extraction of the urine is an important element in quantitation It is essential for making comparisons that extracts be prepared in a standardized manner.

Normal children put out very nearly as much aldosterone each day as normal adults, in spite of the rather marked discrepancy in the production of ketosteroids and smaller difference in the output in 17-hydroxy-corticoids (Porter-Silber chromogens).

In adrenalectomized patients and those with Addison's disease, we see no sodium-retaining activity In the patient with panhypopituitarism, there is virtually normal output of aldosterone, in contrast to the greatly reduced output of ketosteroids and chromogens [15].

When a normal individual received ACTH, the output of hydrocortisone-like and 17-ketosteroids increased by a factor of 5, while an insignificant increase in the output of aldosterone was found in the urine. There was not much change in sodium excretion in this subject, who received a single injection of 80 clinical units of ACTH gel. It is possible that ACTH given under different circumstances might produce some change in the output of aldosterone

In sodium depletion, a different pattern of adrenal cortical function is seen.

When a healthy man reduced his sodium intake from a control level of 120 mEq. per day to 11 mEq. per day, the urine sodium fell to about 4 mEq. per day. At the same time, the aldosterone output rose to 5 times the control level in the 24-hour extract There was also an increase in the glucuronidase extract, while the immediate acid extract showed less striking changes. While these events were taking place, there was almost no evidence of change in the other secretions of the adrenal cortex The 17-ketosteroids were unaffected, as previously reported in patients on low sodium diets. There was no significant change in the output of Porter-Silber chromogens The clearance of inulin and of endogenous creatinine chromogen was not significantly changed during 6 days of sodium deprivation [16].

The serum sodium dropped only slightly, while potassium rose for a day or two. The hematocrit rose There was a modest fall in body weight.

We have the impression here we are seeing a normal mechanism for sodium conservation. I was hoping that Dr Lauson would be here. Henry has often told us that aldosterone is unimportant because if the glomerular filtration rate is low, no sodium reaches the distal tubule for reabsorption I was hoping to point out to him that since the distal tubule completely reabsorbed a normal load in the presence of

[15] Luetscher and Axelrad, J Clin. Endocr. Metab. 14. 1086, 1954.
[16] Luetscher and Axelrad, Proc. Soc Exper Biol. and Med.; in press, 1955.

of increased aldosterone, the filtration rate was immaterial.

It is just a question of which way you want to look at it. Obviously, there is a balance here. If there is an acute reduction in filtration rate, there would be a corresponding acute fall in output of sodium until the kidneys and adrenals had time to compensate. This readjustment, which, we believe, includes adrenal function, allows for adaptation to chronic changes in glomerular filtration rate.

We have described changes in aldosterone output during spontaneous or induced diuresis in nephrosis [17,18]. The output of sodium-retaining hormone in nephrosis was increased. The initial high output of aldosterone occurred with a normal serum sodium concentration, as well as in those patients with hyponatriemia When ACTH was given, and when diuresis followed, there was a sharp fall in the output of sodium-retaining activity of the urine. McCall and Singer [19] noted similar changes after ACTH. They also measured formaldehydrogenic steroids, which showed a rather sluggish increase after ACTH, as expected

Similar results were observed. in the post-cortisone diuresis. With the fall in body weight, with elimination of water and sodium in the urine, the high, initial level of sodium-retaining activity in the urine fell toward normal. This change in the sodium-retaining activity reflects changes in aldosterone in urine.

We have seen comparable changes during diuresis on cortisone, after cortisone, during ACTH administration, or after the withdrawal of ACTH. These findings did not suggest direct effects of ACTH on adrenal cortical secretion of aldosterone, but rather indirect effects of the anti-inflammatory hormone, producing general improvement of the patient's condition

In the patient who has the nephrotic syndrome, but who is temporarily in an edema-free phase, changes are seen in aldosterone output after administration of ACTH. A young man with the nephrotic syndrome, without edema, was treated with ACTH gel in an unsuccessful effort to modify the proteinuria. On the second and third day of therapy, there was a fall in sodium excretion from the control level of 135 to 7 mEq. per day, followed after one week by release of sodium in the urine, and loss of fluid which he had accumulated during the early days of therapy. The output of aldosterone was at a normal level initially, rose sharply on the second and third days, corresponding with the fall in the urine sodium and fell again to subnormal levels during diuresis.

Obviously, we are not dealing with any simple relation between ACTH and aldosterone output, although the expected rise in 17-ketosteroid and Porter-Silber chromogens appeared and was maintained during ACTH administration.

The patient was on a normal sodium intake during these observations. When his urine sodium dropped to 7 mEq. per day, he retained approximately 130 mEq. per day

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[17] Luetscher and Deming, J. Clin. Invest. 29: 1576, 1950.
[18] Luetscher, Deming, and Johnson, J. Clin. Invest. 30: 1530, 1951.
[19] McCall and Singer, J Clin Endocr. Metab. 13: 1157, 1953.

and accumulated water at a corresponding rate without significant change in the serum sodium concentration.

When a patient received cortisone for five days, without apparent effect on his steadily increasing weight or on the low output of sodium, there was no change in the assay of sodium-retaining materials in the urine. It has been found consistently that when treatment fails to relieve edema, it also fails to affect the output of sodium-retaining activity in the urine. This is equally true with ACTH [17, 18].

A number of patients were also treated with concentrated human serum albumin [18] With diuresis and release of sodium in the urine, there was a fall in sodium-retaining activity to normal levels. These data indicate that the output of aldosterone is more closely related to fluid balance and the factors controlling this balance, than to control by the anterior pituitary corticotropin of the conventional type.

This is obviously a complicated situation, and we have a great deal more to learn. To summarize our position at the moment, we believe that aldosterone regulates the sodium output, but we are not prepared to state in simple terms what regulates the output of aldosterone. The secretion of aldosterone appears to be a normal mechanism for sodium conservation. It can be stimulated by sodium deprivation. The output of aldosterone increases in several diseases during the accumulation of edema, when the increased output of aldosterone is associated with very low output of sodium in the urine. We do not believe that this is merely a reaction to sodium depletion occurring in the course of disease, because we have pushed the sodium intake to high levels, without affecting the increased output of aldosterone

Corticotropin has little effect on the output of aldosterone in normal men, but there may be considerable effects in disease

Perhaps, some teleological speculations may be permissible at this point. I have repeatedly emphasized that the patient with the nephrotic syndrome must dilute his plasma in order to stay alive If he tried to maintain a normal plasma colloid osmotic pressure with his greatly reduced quantity of circulating protein, his plasma volume would be so small as to make life impossible A similar situation may exist in hepatic cirrhosis, complicated by diversion of fluid into the peritoneal cavity If we exchange blood flow for blood volume, cardiac failure might be included.

We still have to consider the possibility that there is a failure of hepatic destruction of aldosterone, resulting in increased blood and urine levels, rather than an increased output by the adrenals

I hope that Dr. Farrell and Dr. Sayers will have more to say about these matters, since their physiological experiments can give simple and clear answers to some of these problems.

Dr Bertha Singer, who worked with Dr. Stack-Dunne last year, may have some comments on the regulation of aldosterone in the experimental animal.

DR. McCRORY: I would like to have you explain in more detail the meaning of sodium loss by the bio-assay method. You stated that you could observe sodium excretion under certain circumstances and that cortisone had this effect. Is this salt excretion effect of cortisone quantitatively important in the sense of influencing the estimation of salt-retaining material acting? In short, does the presence of cortisone negate or lessen the salt-retaining response of the assay preparation. If this occurs the salt-retaining activity would be lessened if the patient were excreting cortisone in addition to salt-retaining steroids. The inability to find increased salt-retaining action in urine extract of patients receiving ACTH or cortisone is not in keeping with the clinical observation of salt and water retention in these patients. Could the bio-assay measurement be influenced by the excretion of cortisone?

DR. LUETSCHER: Dr. McCrory's comments raise questions, first about bio-assay, and second, about patients.

Let's take these one at a time very briefly. In the bio-assay, we are dealing with adrenalectomized animals, which have subnormal filtration rate and impaired ability to excrete water after loading. Dr. Sala has studied renal function under the conditions of the bio-assay [20]. When small amounts of aldosterone are given, there is no change in filtration rate, but tubular reabsorption of sodium is increased. when corticosterone, hydrocortisone, or cortisone is given, there is an increase in filtration rate, and the impaired diuresis of the adrenalectomized, water-loaded animal is abolished. A large volume of urine appears, together with an increased output of sodium and potassium. This effect can be eliminated by reducing the water load. For technical reasons, we have found it inconvenient to do so It is also useful to have a qualitatively different response to aldosterone. The quantity of cortisone present in the urine is not enough to affect the assay, as we have shown.

In man, we have to consider again the double effect of these hormones on filtration rate and on tubular reabsorption The patient with the nephrotic syndrome is in many ways not unlike our adrenalectomized rat because both have a subnormal filtration rate, which can be increased by the administration of cortisone or hydrocortisone, together with impaired diuresis. However, the over-all effect of the hormone on the patient depends on a very complex series of phenomena, which include changes in filtration rate, tubular function, adrenal cortical secretions of several types, diet, metabolic changes, water intake, diuresis, and changes in the pathologic physiology of the disease It is tiring to contemplate this over-all picture. If you try to analyze these factors as clearly as you can, the situation may not be so complex or so difficult to understand as it appears on first glance. I have emphasized some consistent features which appear regularly in our series of observations.

DR. MILTON RAPOPORT: Several years ago at the Boston Conference, I mentioned a rather puzzling situation in a child with the nephrotic syndrome. A small boy who had all the findings of the nephrotic syndrome -- edema, low serum proteins, hypercholesterolemia -- but no proteinuria! His swelling had been recognized four days before he entered the hospital. He was quite edematous and continued so for five days, following hospitalization. An accompanying upper respiratory tract infection was treated with penicillin injections, -- no other therapy was used. Following the fifth day he diuresed and lost his edema completely in two days. Urinalysis was carried out 14

[20] Sala and Luetscher, Endocrinol. 55: 516, 1954.

times on this youngster and showed no protein with the routine clinical tests, and a maximum of 50 mg. per 12 hours by a quantitative test; a value which we consider normal. His renal function as judged from his NPN concentration and his urinary specific gravity of 1030, was apparently adequate and normal.

Dr. Henry Lauson appeared incredulous. This child's findings did not fit a theory of the genesis of hypoproteinemia which he held, However, this boy proved that he had the nephrotic syndrome, by returning to the hospital two months later with edema and marked proteinuria in addition to the other characteristic abnormalities. Dr. Lauson suggested that this youngster had had proteinuria before we had seen him the first time -- a point which he couldn't prove -- and we couldn't deny. However, Dr. Luetscher has suggested an alternative explanation -- the increased secretion of aldosterone is part of the nephrotic syndrome and it persisted, even though a postulated antecedent proteinuria had disappeared -- and so accounted for this youngster's edema

DR LUETSCHER: A most interesting observation, Dr. Rapoport. I would agree with your conclusions I hope that you don't suggest that proteinuria is primarily responsible for the hypoproteinemia.

DR. RAPOPORT· This is a point I'd rather not get involved in now.

DR BARNETT: I am very sorry Dr. Lauson is not here to state his own opinions.

In regard to Dr. Rapoport's patient, I think Dr. Lauson would have suggested that a period of proteinuria may have preceded the time when the patient had edema without proteinuria

We have recently followed very closely a child whose proteinuria disappeared. The rate at which the plasma proteins increased in this child was really quite slow extending over a period of many days.

In regard to what causes excessive retention of the salt and water in a child with nephrosis, I do not think anybody really has techniques to resolve the question concerning the relative importance of changes in glomerular filtration rate or in tubular reabsorption. Certainly no one would deny that in terms of sodium the final composition of the urine is determined by the tubules. However, I think Dr. Lauson would say, and I would agree, that changes in glomerular filtration rate may influence what the tubule does in determining the final composition.

There are two questions I should like to ask I thought that Dr. Alex Leaf had shown during sodium restriction there was an increase in the excretion of ketosteroids

The other question I had concerns the relationship between sodium excretion and aldosterone excretion. You demonstrated a direct relationship between the two in a variety of patients. I wonder whether or not this relationship might be altered in the same way that the relationship between anti-diuretic hormone and water excretion can be altered under certain abnormal conditions.

We know, for instance, that under maximum anti-diuretic hormone action, one can alter the rate of urine flow by changes in the rate of solute excretion. I think it would be very interesting to know whether you have, or if you haven't, whether you plan to study aldosterone excretion during sodium loading of an anhydremic subject. It would be of interest also to know about aldosterone excretion in a child with active nephrosis who was forced to excrete sodium by chronic "salt-loading".

DR. LUETSCHER: Dr. Leaf [21] showed that there were changes which he interpreted as secondary to adrenal oversecretion at this time, but I don't believe that ketosteroid or corticosteroid excretion were measured. In subsequent studies, Daughaday and MacBryde [22] did not demonstrate any change in the output of ketosteroids or of neutral reducing lipids during sodium deprivation Our results confirm these observations.

With respect to the acute experiments, unfortunately, it requires quite a long urine collection in order to obtain enough aldosterone to measure accurately.

With respect to sodium loading, I am quite sure that with an adequate sodium load, more sodium would appear in the urine, as Dr Fox has shown us. We don't have the crucial data to interpret this observation. Overflow of sodium could occur under either of two circumstances. If you give a constant dose of desoxycorticosterone to a dog or patient, you can still cause him to excrete sodium, if the sodium load is increased sufficiently. The accumulation of body fluids can apparently override the action of a constant dose of that hormone, even in the absence of the adrenals, though with difficulty in the latter case. In the patient whose adrenals are responsive to the internal environment, any excess of sodium accumulated in the preliminary phases might cause a suppression of aldosterone output. It would be extremely interesting to measure aldosterone under these conditions.

DR. ARTHUR MERRILL: I would like to ask Dr. Leutscher if he has correlated aldosterone with the sweat sodium.

DR. LUETSCHER: We have not done so, but in view of Dr. Conn's previous observations [23], there would probably be a correlation. Simpson and Tait have given aldosterone to normal individuals and have shown an elevation of the potassium to sodium ratio in saliva [13]. Berger and Steele [24] noted suppression of sodium excretion by the colon in heart failure and cirrhosis of the liver. Evidently there are effects on cells other than the renal tubules.

DR. MERRILL: ACTH always lowers sweat sodium content, does it not?

DR LUETSCHER: I have no personal experience, but published reports suggest that ACTH lowers sweat sodium content.

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[21] Leaf and Couter J Clin. Invest. 28:1067, 1949.
[22] Daughaday and MacBryde J. Clin. Invest. 29:591, 1950.
[23] a. Conn Arch. Int Med 83:416, 1949.
b. Locke, Talbot, Jones, and Worcester J. Clin. Invest. 30:325, 1951.
[24] Berger and Steele J. Clin. Invest. 31:451, 1952.

DR JOHN JAMES: I should like to ask Dr. Leutscher whether any studies have been done on aldosterone output in animals subjected to plasmapheresis

DR. LUETSCHER: I have heard no reports on this subject. We did a very extensive study some time ago on plasmapheresis in dogs and were able to show that we did not affect the filtration rate significantly, nor did we affect the sodium excretion significantly, even when very marked hypoalbuminemia developed. We published these data only in abstract form, but they were subsequently repeated and confirmed by other observers I consider the story of plasmapheresis in animals to be a sort of bad joke. I spent one whole winter working with Dr. A D. Hall and Miss Kremer in Baltimore trying to make some dogs edematous by plasmapheresis and never really succeeded A protein-free diet is essential. without this, plasmapheresis is futile. If you go back to the original experiments, you will discover that in order to keep the dogs comfortable or happy or something during the procedure, investigators used to infuse something like 10 per cent of body weight in isotonic sodium chloride solution each day during the experiments When the serum proteins are reduced, edema is produced more readily. In the absence of sodium and water loading, edema does not occur spontaneously until the last stages when the dog is dying of malnutrition.

DR MILLER: I think it might be wise to offer a word of caution on the interpretation of bio-assays You mentioned that other salt-retaining substances exist, and until the day comes when easy methods become available to determine which substance, we will not have the final answer You might be interested in a second-hand report from the Central Society Meeting in Chicago last week where the Mayo Clinic people reported giving as much as 2 to 3 milligrams of aldosterone to patients with rheumatoid arthritis over a period of five days. The amount of sodium and water retained was surprisingly small in these otherwise normal individuals Very little effect on potassium excretion was observed I think the whole matter of what aldosterone does in the human still awaits further experimentation.

DR LUETSCHER. I am glad that Dr. Miller has raised these points so that they can be cleared up In our earlier work, we measured sodium-retaining activity of urine extracts Subsequently, it was found that only one fraction of these extracts showed sodium-retaining activity. The active material was proved to be aldosterone in nephrosis, and we believe that the active material of similar properties is aldosterone in the other cases also. When the word, aldosterone, is used, I mean a sodium-retaining substance with chromatographic properties identical with aldosterone

Secondly, in the interpretation of studies in man, we must be exceedingly cautious for a while because the short duration of action of aldosterone in man has not been generally recognized.

Prunty [25] has shown that the effects of aldosterone appear and disappear quickly. If one large dose of aldosterone is given to a man, most of the effects occur in the 4 to 6 hours following the injection. Temporary suppression of sodium output may be almost complete, but in the subsequent hours there is increased sodium excretion

[25] Prunty, McSwiney, Mills, and Smith Lancet 267:620, 1954.

above the baseline. The preparations of aldosterone which are available at the present time are not suitable for sustained action. Until longer acting materials are available, day-to-day balance studies must be viewed with a critical eye.

DR. T. S. DANOWSKI: It might be helpful to keep in mind there are multiple regulators of sodium retention and excretion. As a clinical counterpart of Leutscher's dog, there are the studies of Rosenbaum, et al. [26] Their patients with Addison's Disease maintained on cortisone responded to water excesses and deficits and to variations in sodium loads. These adjustments could not have been mediated through the adrenal cortices, and hence other regulators must have been operative.

Secondly, in support of this view, you might expect that, with a compound such as aldosterone which leads to sodium retention and to potassium excretion, such processes are inherent and inflexible properties of the compound. However, under conditions of complete electrolyte deprivation where the urine becomes sodium free, the rat produces a urine which is also essentially potassium free. If this complete conservation of sodium is mediated through aldosterone, then one might expect an increased potassium output Since this does not happen there must be regulators of potassium other than aldosterone

DR. LUETSCHER: There are mechanisms of the kidney which work in the absence of adrenal steroids and others which will operate if given small amounts of hormone. But it is easy to demonstrate serious deficiencies of renal function in the adrenalectomized animal or man A small dose of cortisone restores some normal reactions, but does not make the Addisonian patient normal, as Dr. Danowski knows as well as any of us.

We think aldosterone is analogous to posterior pituitary hormone, which adds the fine regulation to the primitive mechanisms which exist in the kidney deprived of adrenal support. The situation is obviously complex, and aldosterone is surely not the only thing that controls the excretion of sodium or potassium

CHAIRMAN METCOFF: Thank you, Dr Luetscher for your stimulating comments. Dr. Farrell now will present studies on isolation and identification of steroids obtained from adrenal venous blood.

DR. FARRELL: We have been interested in the past few years in isolating and identifying the steroids in adrenal venous blood. We believe that this is the most accurate way of defining the nature of the secretion of the adrenal cortex. I would like very briefly to present some of our more recent work.

Figure 85 will illustrate a typical experiment in which a sample of adrenal venous blood from the dog, collected by cannulation of the lumbo-adrenal vein, is extracted with chloroform and partitioned between 70% ethanol and hexane. The solutes from the 70 per cent ethanol fraction are chromatographed through a series of paper chromatograms. We employ the solvents propylene glycol-toluene for the separation of the more polar steroids such as 17-hyroxycorticosterone and corticosterone. Propylene glycol-hexane serves well for compounds of the order of polarity

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[26] Rosenbaum, J.D., et al J. Clin. Invest. 31:657, 1952.

Fig. 85

Fig. 85. Flow sheet of analysis of steroids of blood

Fig. 86. Steroids in adrenal venous blood

Fig. 87. Sodium-retaining activity of steroids in adrenal venous blood

Fig. 86

Fig. 87

of 11-desoxycorticosterone. The isolated fractions are routinely studied for absorption in the ultraviolet, reaction with ammoniacal silver nitrate and reaction with phenylhydrazine. Bio-assay for sodium-retaining activity is also routinely employed to aid the isolation of aldosterone.

DR. BARNETT: With how much blood do you start?

DR. FARRELL: This varies with what we have in mind The largest amount of blood we have used was 50 liters, during the initial studies on aldosterone. We ordinarily use about two liters for an experiment

Figure 86 shows the steroids which we have isolated from adrenal venous blood of the dog The two largest components are clearly 17-hydroxycorticosterone and corticosterone. In the region of the chromatograms corresponding to higher oxygen levels on the steroid nucleus are found five components which have not been identified. Slightly less polar than 17 hydroxycorticosterone is an unidentified steroid, Fraction 7 This summer Elizabeth Rauschkolb, Paul Royce, Hans Hirschmann and I were able to isolate and identify aldosterone from this source[27]. 11-Desoxy-17-hydroxycorticosterone is present. In the less polar regions of the chromatograms are four components, one is probably 11-dehydrocorticosterone, the second is unidentified, the third is 11-desoxycorticosterone and the last is unidentified 11-Desoxycorticosterone has only recently been identified as a component of this secretion[28]

The finding of these steroids in the adrenal venous blood itself does not, of course, mean that the adrenal secretes them. It was necessary to study venous-arterial concentration differences It was found that the five steroids more polar than 17-hydroxycorticosterone (that is, probably more highly oxygenated) are not directly secreted by the adrenal since they are found in the peripheral blood in the same concentration as in adrenal venous blood. They may well be degradation products of the major steroids The same is true of Fraction 7. An occasional animal does not secrete Substance S. The other steroids appear to be secreted by the adrenal, as evidenced by these A-V difference studies

This work clearly indicates that the adrenal secretes a complex mixture of steroids The question naturally arises as to the relative importance of these steroids in the metabolic balance of the animal. If we consider the effects of the steroids on carbohydrate metabolism, there appears to be little question the 17-hydroxycorticosterone is the most important steroid in this regard. It is, so far as we know, the most potent carbohydrate-active steroid in the secretion and is present in the highest concentration With regard to electrolyte metabolism, the situation is more complex, since aldosterone, corticosterone and desoxycorticosterone all play a role. The relative sodium-retaining activities of these fractions has been estimated (Fig. 87) from our data on the relative concentrations of the steroids in adrenal venous blood and

[27] Farrell, G. L., Royce, P. C., Rauschkolb, E W. and Hirschmann, H., Proc. Soc. Exp Biol. & Med., 87. 106, 1954.

[28] Farrell, G. L., Royce, P. C., Rauschkolb, E. W. and Hirschmann, H., Proc. Soc. Exper. Biol. & Med., In Press.

and from the literature. This is a representative sample of adrenal venous blood obtained from animals on a moderate intake of sodium chloride. 17-Hydroxycorticosterone contributes 3 per cant and corticosterone 27 per cent of the sodium-retaining activity. Aldosterone is the most important component since it is responsible for 69 per cent of the sodium-retaining activity of the sample. This is true in spite of its comparatively low concentration because of its very high potency. Desoxycorticosterone contributes very little (about 1 per cent) of the total activity.

It was of interest to determine which of these steroids was under pituitary control. The experiments shown in Fig. 88 were conducted in the following manner. Dogs were hypophysectomized via the oral approach. Six hours later the adrenal vein was cannulated and from the sixth to tenth hour adrenal venous blood was collected. This blood was analyzed for steroid components. The cross-hatched areas represent the control levels, the clear areas, the hypophysectomy levels of steroid secretion. I think it is fairly evident that each of the steroids is influenced by hypophysectomy. The effect is quite profound with regard to the secretion of 17-hydroxycorticosterone, corticosterone, 11-desoxy-17-hydroxycorticosterone and 11-desoxycorticosterone. The rate of secretio of aldosterone seemed to decrease less proportionately than the others. In order to further study this problem we conducted an additional series of experiments in which we isolated aldosterone following hypophysectomy. Figure 89 shows the results of experiments conducted on thirty-two animals, sixteen sham hypophysectomized and sixteen hypophysectomized. The cross-hatched bars show the levels of secretion of the controls and the clear, those of the hypophysectomies. It is evident that the levels of *secretion of 17-hydroxycorticosterone, 11-desoxy-17-hyroxycorticosterone and corti-*costerone fell to about one tenth of the control during this period following hypophysectomy. The level of secretion of aldosterone consistently remained at somewhat less than one half the control level This finding suggests either that the time relationship of the decrease of the secretion rate of aldosterone following hypophysectomy is different than for the other steroids, or that aldosterone is synthesized by two pathways, one ACTH dependent, the other not.

The effect of ACTH injected intravenously into hypophysectomized dogs is shown in Figure 90. The levels of secretion of 17-hydroxycorticosterone, 11-desoxy-17-hydroxycorticosterone, corticosterone and 11-desoxycorticosterone by the adrenal of the hypophysectomized dogs (cross-hatched areas) rise sharply following ACTH(Clear areas). Fractions 13, 13A and 14A have not been isolated in a study of this kind. The increase in the rate of secretion of aldosterone following ACTH is less than it is in the case of the other steroids. This raises once again the question as to the extent of the pituitary regulation of this important steroid component.

CHAIRMAN METCOFF: Thank you, Dr. Farrell. I wonder if we can proceed to Dr. Sayers' presentation and then discuss both afterward.

DR. GEORGE SAYERS. I have no experimental data of my own to present. I want to take this opportunity to compare the metabolic actions of hydrocortisone, corticosterone, aldosterone *and desoxycorticosterone.* I will emphasize at the outset that aldosterone is not merely a more potent form of desoxycorticosterone. We now have experimental data from various laboratories which indicate quite clearly that aldosterone has biological features of its own which distinguish it from hydrocortisone and from desoxycorticost-

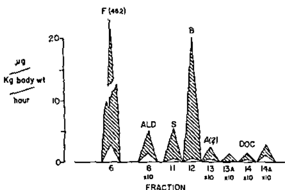


Fig 88

Fig. 88. Effect of hypophysectomy on steroids secreted by the adrenal

Fig. 89. Secretion of adrenocortical steroids after hypophysectomy

Fig. 90. Effect of ACTH on rate of steroid secretion by the adrenal

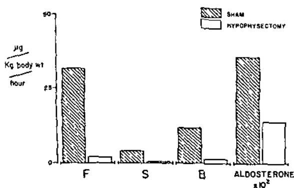


Fig. 89

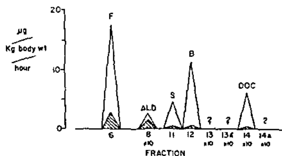


Fig. 90

erone. If time permits, we may consider some wild schemes on what possibly is going on in the nephrotic syndrome in regard to excretion of aldosterone in the urine. Why the excess of this steroid? What mechanism regulates the rate of secretion of aldosterone? By what means does ACTH and cortisone reduce the output of aldosterone in the nephrotic syndrome?

I have gathered from the literature experimental data on the metabolic actions of hydrocortisone, corticosterone, aldosterone, desoxycorticosterone (see Table 34). The four steroids are of special interest as far as adrenocortical physiology is concerned

Dr. Farrell has established the fact that hydrocortisone, corticosterone, aldosterone, desoxycorticosterone are elaborated by the dog adrenal cortex, and Dr. Sweat in our laboratory has good evidence that hydrocortisone and corticosterone are elaborated by the human adrenal.

In Table 34 I have given the most potent compound a rating of 100. The comparisons are made on a weight basis, that is, the activity of a unit weight of hydrocortisone is compared with the same weight of each of the other three steroids Hydrocortisone is much more potent than desoxycorticosterone in promoting deposition of glycogen. Aldosterone has an appreciable effect on carbohydrate metabolism and is, therefore, quite distinct from desoxycorticosterone.

However, from what we just heard from Dr Farrell, the quantity of aldosterone secreted by the adrenal cortex is so relatively small that obviously hydrocortisone is the important steroid as far as the regulation of carbohydrate metabolism is concerned.

Life maintenance I have divided into two categories. optimal conditions and stressful conditions By optimal conditions I mean a situation in which the animal is maintained at a fairly constant temperature and free from infection. The sodium chloride intake of the animal is that of a regular diet There has been no supplementation of sodium chloride

Aldosterone is, by far, the most potent compound. Desoxycorticosterone is one twenty-fifth as potent Hydrocortisone is very weak in this regard The well-being of the animal under optimal conditions is in large measure determined by aldosterone. The situation changes when we talk about survival under stressful situations. Here you see that, whereas hydrocortisone was very weak in comparison to desoxycorticosterone under optimal conditions, hydrocortisone becomes very important under stressful conditions. (The rating in Table 34 is for cortisone, but I am quite sure the same rating applies to hydrocortisone) So here is a different type of activity being revealed when the animal is under a stressful situation

I have picked two types of stresses, cold and exercise, and in both situations hydrocortisone is much more potent than desoxycorticosterone

Now, the data on aldosterone are a bit fragmentary yet, but from what information we have it appears that aldosterone is as potent as hydrocortisone under conditions of cold stress.

TABLE 34*

	Hydrocortisone	Corticosterone	Aldosterone	Desoxycorticosterone
Carbohydrate Metabolism				
Liver glycogen	100[5]	30[5]	25[11]	1[5]
Life Maintenance				
Optimal conditions	0.05[12,17]	+	100[10,12]	4[10,12]
Stress				
Cold	100[7] (for E)	9[7]	100[8]	8[7]
Exercise	100[6]	30[6]	?	1[6]
Renal Function				
NPN	0.05[4]	2[4]	100[12]	4[4]
GFR Rat	Increase[16] (for E)	?	No change[16]	Slight increase[16]
GFR Dog	Increase[18]	?	?	No change or slight increase[1,15]
Inhibition of ACTH Discharge	100[14]	25[14]	33[13]	10[14]
Eosinopenia	100[19]	25 (for A)[19]	50[8,9]	1[19]
Electrolyte Metabolism				
Na^+ Low Na^+ reserves	Retention = 0.05[17]	Retention = 1[18]	Retention = 100[3,12]	Retention = 4[3,12]
Normal or high Na^+ reserves	No retention or excretion[1,15,20]	No retention[2]	?**	Retention[2,21]
K^+ excretion	Increased	Increased	100[3]	20[3]
Water excretion after water load	++++	?	+[10]	+[10]

* Superscript numbers refer to references on facing page

** Dogs maintained on DOCA. Switched from DOCA to aldosterone. Transient increase in Na^+ excretion[10]

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We have no information on the potency of aldosterone in the exercise test of Ingle.

It looks as if hydrocortisone is the important steroid in resistance to noxious stimuli. Now we shall consider renal function Aldosterone is by far the most potent steroid in maintenance of normal renal function as measured by blood NPN; desoxycorticosterone is about one-twenty-fifth as potent.

It seems the effect on NPN about parallels the sodium-retaining effect. Hydrocortisone is relatively inactive under the conditions of the particular assay system that I am describing here. Corticosterone is also quite weak.

Hydrocortisone apparently increases glomerular filtration rate in the rat, in the dog, and in man. This is an important aspect of the problem of the effect of these steroids on sodium excretion. Dr Luetscher has pointed out that sodium load may be increased by steroids which increase GFR.

Desoxycorticosterone may have some effect on glomerular filtration rate, but the effect doesn't seem to be nearly as pronounced as in the case of hydrocortisone.

Dr Luetscher and Dr. Sala have some data on the effect of aldosterone on GFR in the rat

It appears that it has no significant effect on glomerular filtration rate in this species. We have no information on the dog or man.

As far as inhibition of ACTH discharge is concerned, hydrocortisone is ten times as potent as desoxycorticosterone. This is an action in which we test the ability of an injected steroid to inhibit discharge of ACTH upon application of a noxious stiumulus. Aldosterone is one-third as active as cortisone. Corticosterone is one-quarter as active. It is obvious from the relative amounts of steroids secreted by the adrenal that the most important steroid inhibiting ACTH discharge is hydrocortisone.

Hydrocortisone is much more potent than desoxycorticosterone in inducing eosinopenia. Aldosterone has an action quite comparable to that of hydrocortisone. Here again we have an indication that aldosterone is distinct in its actions from desoxycorticosterone.

I anticipated considerable difficulty in discussing the role of the adrenal cortex in sodium excretion. This is a very complex problem, indeed, as Dr. Luetscher pointed out. I would like to add to the discussion that has gone before to say that it is a multi-dimensional problem in the sense that it is not only a matter of the dose of steroid, but it is also a matter of the sodium reserves in the animal. When one describes the effect of hydrocortisone on sodium retention, one must be careful to state the dose of the steroid and also the amount of sodium in the animals' diet.

As far as retention goes, on low sodium reserves, aldosterone is the most potent compound; it is twenty-five times as potent as desoxycorticosterone. From Swingle's

work on adrenalectomized dogs it appears that on relatively low sodium intake, hydrocortisone has 1/2000th the activity of aldosterone. Very weak activity, indeed!

On normal or high sodium reserves, one sees little or no retention, sometimes even excretion following the administration of hydrocortisone. This is quite distinct from desoxycorticosterone, which regardless of the sodium intake, be it low or be it high, induces retention. There appears to be a qualitative difference between hydrocortisone and desoxycorticosterone.

We are beginning to get some experimental data on the effect of aldosterone under the circumstances of normal or high sodium reserves. I am not willing to make a final statement at this time as to just what the action of aldosterone will be in high doses in an individual on moderate or high sodium loads.

The Swiss investigators, Gross and Gysel, maintained adrenalectomized dogs on desoxycorticosterone The day following the withdrawal of desoxycorticosterone the animals were given aldosterone, and for the first few days on high dose of aldosterone increased sodium excretion occurred.

There is a gap in our knowledge regarding the action of aldosterone under circumstances of high sodium intake that obviously has to be filled

This knowledge is quite important as far as the role of aldosterone in the nephrotic syndrome is concerned.

Potassium secretion may be discussed briefly Aldosterone is five times as potent as desoxycorticosterone. The effect of the steroids on water excretion is interesting. As you know, the patient with Addison's disease is quite incapable of handling a water load Desoxycorticosterone has a slight effect in increasing the rate of excretion of water. Hydrocortisone, on the other hand, has a very marked effect and can restore the deficient organism to normal at relatively low doses.

It is rather interesting that aldosterone falls into the same catergory as desoxycorticosterone in this regard

CHAIRMAN METCOFF: Thank you for this enlightening presentation Dr. Sayers. I wonder if Dr Singer might like to comment at this point

DR. BERTHA SINGER I know there isn't much time, so perhaps I will get right down to the point, if I could show just one slide, summarizing some of the work I did last year in collaboration with Dr. M.P Stack-Dunne on the adrenal vein blood of rats studied under a variety of experimental conditions.

First, a word about the collection and handling of the blood. It was collected from the adrenal vein, and extracted with ethyl acetate and chromatographed. Corticosterone was estimated by the fluorescence reaction on paper The aldosterone was eluted from the appropriate paper chromatogram fraction and assayed biologically by the Simpson and Tait assay [29]

[29] Simpson, S. A. and Tait, J F. Endocrinology. 50:150, 1952

We have plotted the values that were obtained in these experiments logarithmically, so that changes which might not appear great at first glance are really quite significant (Fig. 91).

The corticosterone is plotted here, and the aldosterone values, were expressed as the DOCA equivalent, in micrograms, per adrenal, per kilogram body weight, per hour.

The blood was collected for an hour and a half in each instance. As you can see, the intact rats secreted quite high amounts of both compounds, and the proportion of the aldosterone to corticosterone was about 0.3 After hypophysectomy, the values of both substances fell, in the case of the corticosterone very dramatically, and the mean aldosterone values of several groups was about one-fifth the values found in the intact animals. These values did not fall in the period during which we studied them, from two days after hypophysectomy to forty-four days.

We then studied the effect of an acute intravenous injection of ACTH given two days after hypophysectomy.

As you can see, both substances shot up to the level found in intact animals, and the ratio of both substances was about the same as in intact animals. However, seven days after hypophysectomy we noted that the aldosterone still responded to the acute injection of ACTH whereas, the corticosterone did not However, when we maintained the animals with ACTH over the whole seven-day period, and then gave them ACTH just before collection of adrenal vein blood, both values seemed to be up in the normal range, although not quite as high as those observed in the intact animals.

From these, we have concluded that the secretion of both substances can go on without the pituitary, and that ACTH can affect the secretion of both. However, there is a marked difference between the dependence of corticosterone on ACTH and of aldosterone on ACTH.

Another set of experiments performed on intact animals, was the effect of DCA treatment on the secretion of both substances and of the effect of a low potassium diet for twenty-eight days. In both instances we found a specific decrease in the aldosterone. Both of these values are less than 90 per cent of the original values, whereas, the corticosterone values were well within the range of intact animals. When we tried a combined experiment on hypophysectomized rats, ACTH maintenance did show an increase in the corticosterone and DCA treatment at the same time did result in the specific inhibition of the secretion of aldosterone in the absence of the pituitary.

I don't know if these experiments will help in understanding what happens in the nephrotic syndrome, but I think they might be of some interest.

Incidentally, we did feed animals on what we thought was a low sodium diet for twenty-eight days, and did get an increase in the secretion of aldosterone, but it was not significant and on analysis of the diet we were quite relieved to find that we had not actually produced very great sodium deficiency. The intake was only cut by 50 per cent.

CHAIRMAN METCOFF: Dr. Barnett.

DR. BARNETT: I had one question I would like to ask Dr. Sayers. What are his speculations about nephrosis?

DR. SAYERS: As I see it at the present time, there are three possibilities that we can think of. Probably the right answer is the one we haven't thought of Dr. Luetscher has mentioned degradation of the steroids.The liver is probably the most important organ in corticosteroid degradation. It is possible that there is some defect in the liver in the nephrotic syndrome which leads to impairment in degradation of aldosterone. This would explain the increased rate of urinary excretion of this steroid. Cortisone and ACTH reduce the rate of excretion of aldosterone by correcting the underlying pathology in the liver.

The second possibility would be the matter of excretion. It doesn't seem likely, but we should consider the possibility that there is something peculiar about the renal handling of aldosterone in the nephrotic syndrome.

Practically no work has been done on the renal handling of steroids. How much steroid is filtered? How much is re-absorbed by the tubule? We don't know how much of these steroids are protein bound These are problems which can be solved. We have the techniques available at the present time.

A third possibility would be some defect in the adrenal cortex.

I don't think there is a likely possibility of a primary defect in the adrenal cortex. This would be a situation analogous to the adrenogenital syndrome in which there is an excessive secretion of an androgen. In the nephrotic syndrome the adrenal cortex would secrete an excess of aldosterone because of an aberration in the metabolic pathways concerned with the biosynthesis of the corticosteroids.

I think it is pretty obvious from what I have learned from you people who know so much more about the nephrotic syndrome than I do, that the basic, underlying defect is probably to be found at a site other than the adrenal cortex.

Perhaps the adrenal cortex is influenced by the status of sodium metabolism. It is pretty obvious from the data we have at the present time that it is not a simple matter of concentration of sodium in the extracellular fluid.

I talked to Dr. Luetscher about potassium, and he feels there is not much evidence to support the notion that the concentration of potassium in extracellular fluid influences the secretory pattern of the adrenal cotex. A change in concentration of potassium doesn't seem to be the cause of the increased output of aldosterone in the nephrotic syndrome. If electrolyte influences the output of aldosterone the mechanism involved eludes us at present

Now, if it is the pituitary, and I have doubts that it is, then one has to modify our present thinking in regard to the pituitary regulation of the adrenal cortex. We now

must think of the elaboration of ACTH, which stimulates the output of F, of B, and of DOC, and in addition an X factor which stimulated the output of Aldosterone.

Of course, this X factor does not fit with Dr. Luetscher's study on the patients with pan-hypopituitarism. You will remember that in these individuals output of aldosterone was quite normal.

The data of Dr. Farrell and of Dr. Singer would indicate the pituitary does have some degree of control. We must introduce another concept to resolve this dilemma.

Now, why does the administration of ACTH and cortisone reduce the output of aldosterone in the nephrotic syndrome? The injection of ACTH would increase the output of the complex, hydrocortisone, corticosterone and 11-desoxycorticosterone. This complex would act back on the pituitary, and here we have to bring in a new concept that this complex not only inhibits the output of ACTH but also X, and that the X factor is a more sluggish system than the ACTH system.

DR. LANGE: Ten days sluggish.

DR. SAYERS: We have no exact experimental data on this point. Dr. Farrell has analysed dog adrenal venous blood six to ten hours after hypophysectomy.

Well, now, let's get back to the business of Dr. Luetscher's data on patients with pan-hypopituitarism. These data do not fit with the scheme of regulation just presented We talked about this problem in the lab yesterday afternoon--Dr. Farrell, Dr. Luetscher and myself, and we came up with a wild scheme. Let's skip the pituitary and go to the hypothalamus. Maybe the steroids inhibit the hypothalamus, and the X factor is not elaborated by *the pituitary at all, but by the hypothalamus.*

That would resolve the difficulty as far as Dr Luetscher's data are concerned, and might also explain Dr Farrell's and Dr Singer's results on the time relationship.

While talking about this, John Luetscher brought up the matter of central mechanisms that effect sodium metabolism. Pretty darn vague, this whole business. But it would help clear the vagueness if it turns out that the central mechanism which is said to influence sodium metabolism is a hypothalamic factor which stimulated the adrenal cortex to secrete aldosterone

Well, so much for the dreaming, it may raise some discussion.

CHAIRMAN METCOFF: I believe Dr. Gallan in Cuba has done hypothalamic sections in nephrotic children, somewhat along this line of reasoning, and as I vaguely recall the data, there was no diuresis in two or three such children, and in one instance, diuresis occurred after ACTH.

DR. BARNETT: There is evidence, as you know, that there is an increase in anti-diuretic hormone in the blood of children with nephrosis.

DR. SAYERS: That brings up the touchy point about the bio-assay of anti-diuretic

hormone. Dr. Van Dyke at the Laurentian Hormone Conference in September emphasized that the methods used for the bio-assay of ADH in plasma are in general worthless since they involve the intraperitoneal or subcutaneous administration of unknown material. The intravenous administration of the unknown material is the only way to assay for ADH Intravenous administration in the intact dog is the method of choice.

DR. BARNETT: This was done in intact man

DR. SAYERS: Injected intravenously? If that is true then your results are truly reliable

DR BARNETT: Just once, in one observation, so I think the results remain in some doubt.

DR LUETSCHER: I believe that Dr Barnett's intravenous assay in man has Dr. Van Dyke's blessing, to judge by his remarks at the Laurentian Conference.

CHAIRMAN METCOFF. We are grateful to all participants for an exciting session. We shall proceed to a discussion of therapy, and Dr. Barnett will assume the chairmanship

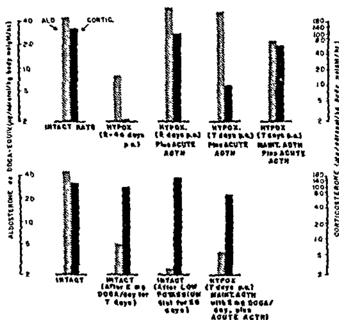


Fig 91 Aldosterone and corticosterone in rat adrenal venous effluent

VI. THERAPY OF THE NEPHROTIC SYNDROME

CHAIRMAN BARNETT. We plan this afternoon to talk about therapy of the nephrotic syndrome

There are a number of people representing different groups whom we are going to ask to present their ideas concerning therapy, within a period of ten minutes each We thought we would not have discussion until the end of these presentations. Because of time limitations, data from other members of the group will have to be presented in the discussion period. Drs Metcoff, Riley, Danowski, Greenman, Rapoport, Spector, Lange, Kramer, Merrill, Taylor, Startzman and McCrory will be asked to present their therapeutic results. We will begin with Dr. Rapoport.

DR MILTON RAPOPORT· In this report, I am presenting an analysis of the results of treatment with cortisone and ACTH of children with the Nephrotic Syndrome at the Children's Hospital of Philadelphia, since 1949. Following the initial demonstrations that intensive corticoid therapy could produce a remission of the Nephrotic Syndrome, many modifications of the original short courses (8 to 12 days) of therapy have been introduced In general, the different regimens are engendered by two shortcomings or failures of corticoid therapy, viz.,

(1) *The failure to bring the Nephrotic Syndrome* under control in some children with relatively large doses of cortisone.

(2) The recurrence of the nephrotic syndrome following remission in many treated children.

Therapeutic investigation with ACTH and cortisone has accordingly followed two lines-

(1) *Short term or "intensive" treatment of the child with nephrosis with* relatively large doses until remission occurred.

(2) Long term or a "maintenance" treatment in an attempt to hold the disease under control.

Whether these two directions of therapeutic effort represent two distinct phases in the evolution of treatment of the nephrotic syndrome with corticoids is not as clear as it would appear at first glance.

It has been our belief that adherence to one plan of therapy would in time give us a large enough body of data from which it might be possible to evaluate the modifying effect of corticoid therapy upon the ultimate course of Nephrosis. Accordingly we have adhered strictly to intensive or short term therapy in edematous patients and have given no maintenance treatment during edema free periods. Even though we were aware that other clinics were experimenting with maintenance therapy we have resisted the oft-times strong temptation to change. No fixed rules dictated the repetition of a course of therapy. When we believed that a particular patient's recurrent edema was becoming burdensome to him, intensive treatment was repeated. In many instances, spontaneous diuresis with disappearance of edema occurred.

In Figure 92 is depicted graphically the clinical status of our patients at increasing intervals of time after their first course of treatment.

The clinical status of the child with the Nephrotic Syndrome may be placed in any of four categories, (which we have indicated by the numerals- I, II, III, and IV - in the circles of Figure 92):

Category I--includes patients who are clinically and biochemically normal--i.e. have neither edema nor proteinuria and have normal renal function and blood chemistry.

Category II--includes patients whose sole abnormality is a trace of protein in the urine.

Categories I and II we have combined and are depicted as a single area in each circle of Figure 92.

Category III-includes patients with no edema but with proteinuria of marked or significant degree.

Category IV--includes patients with edema in addition to proteinuria and biochemical changes in the blood.

In the upper portion of Figure 92 is shown the clinical behaviour of 40 children for a one year period after an initial single course of short duration (8 to 12 days) of either ACTH, cortisone or compound F. In 1949 and 1950 ACTH was employed at a dosage of 2 mg per pound per day From 1951 through 1954, cortisone and compound F (5 mg. per pound, per day), have replaced ACTH.

At the beginning of treatment all children were markedly edematous, i.e. were in Category IV. The first circle shows the immediate response to intensive corticoid therapy. Approximately three quarters of the children (77.5%) lost their edema (Groups I, II and III combined), but only 45% (*Groups I and II combined*), *of the patients stopped* excreting protein. The gradual reversion of most of the patients to their pre-treatment status is shown by the circles showing the successive distribution of categories at 2, 6 and 12 months following initial treatment. At one year, but one in fourteen patients was in the group that we considered favorable (i.e. Categories I and II combined).

CLINICAL STATUS AFTER INITIAL

COURSE STEROID THERAPY

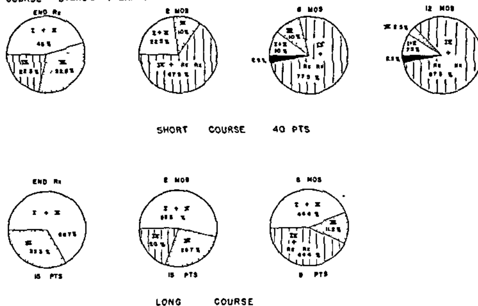


Fig. 92. Clinical status after initial course steroid therapy

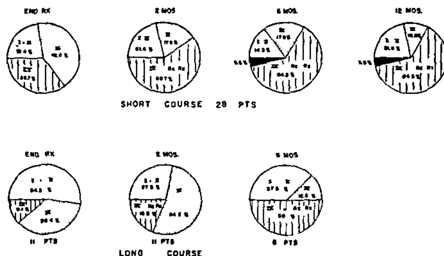


Fig. 93. Clinical status after repeat course steroid therapy

In the lower half of Figure 92 is depicted the clinical behaviour of a group of 15 patients treated more intensively with a single course of cortisone; for 28 days in contrast to the short 8 to 12 day courses employed in the preceding 40 patients. The extension of the period of cortisone administration to 28 days was prompted by the enthusiastic report by the University of Pittsburgh group of their results with 28 day ACTH administration at the 1953 Conference on the Nephrotic Syndrome in Philadelphia. The status of our patients is depicted up to 6 months after treatment. The number followed for one year is too small for analysis.

The striking differences in response of this group of 15 patients treated for 28 days compared to those of the 40 children given 8 to 12 day courses are obvious. All 15 children lost their edema, whereas 22.5% of the short course patients did not. Ten of the fifteen (66 7%) were in almost complete remission,(Group I and II), i.e. free from edema and proteinuria immediately after therapy as compared to 45% of the children treated for the shorter period. Furthermore, remission continued for a longer time following 28 day therapy. At 6 months 44 4% were in Group I and II, while only 10% of the short course patients were in this group at the same interval.

In Figure 93 are depicted the clinical conditions of 28 children followed for a period of one year after a second short course (8 to 12 days) of cortisone All were edematous (in Category IV) before treatment. There was greater incidence of therapeutic failure (35 7%) than after the first course (22 5%) Similarly, the favorable group (Categories I and II combined) was smaller,--21 4% as compared to 45%. At 2, 6, and 12 months after treatment the reversion of these children to the less favorable Categories III and IV paralleled the behaviour of the patients after their first course of cortisone.

In the lower half of Figure 93 are shown the responses of eleven children to a second 28 day course of cortisone. In general, the therapeutic results are better and longer lasting than those in the group given the second shorter course. Using the clinical status of patients six months after therapy as a criterion, a second 28 day course of cortisone appears to be as effective as the first course.

The varying responses, both immediate and ultimate, of children with Nephrosis to steroid therapy which are depicted in Figures 92 and 93, led us to search for factors which might have some bearing on this variability. During the course of steroid administration measurements of daily urinary excretion of protein were done Depending upon whether proteinuria persists or disappears under the influence of steroid therapy, we have divided our children into two groups.

In Figure 94, data are presented on 22 children (Group I) who lost their proteinuria while receiving steroids Diuresis, with resultant loss of edema occurred in every child, and had its onset from the fifth to the twelfth day of therapy. Urinary loss of protein which averaged 3 grams daily at the onset of therapy, declined to low levels by the eighth day, and had ceased by the fourteenth day. Continuation of steroid therapy beyond 14 days had no additional effect.

In Figure 95, similar data are depicted for 13 children who did not lose their

proteinuria with steroid therapy, even though 8 children developed diuresis with loss of edema. The line depicting the average urinary loss of protein per 24 hours, shows a slow fall from an initial value of 6 grams to 3 grams on the tenth day of treatment, and a gradual rise in urinary protein excretion with the continuation of therapy, to levels of 4.5 grams on the twenty-third day of steroid administration. The more massive proteinuria with continued therapy is curiously not indicative of deterioration of the patient, but is a result of further improvement of his status, i.e. with a glomerular leak of fixed magnitude, the increased proteinuria is due to higher levels of circulating serum albumin.

Based on the influence which steroid therapy exerts on proteinuria, we feel justified in speculating somewhat on the prognostic value of this response in individual patients. It is reasonable to believe that the renal lesion at the onset of Nephrosis varies quantitatively from patient to patient. The degree to which an adequate course of steroid therapy (14 days from our data) abolishes the glomerular leak responsible for proteinuria would appear to be an index of the severity of the lesion at the time of measurement. It would seem that the patient who continues to have proteinuria after 14 days of steroid in adequate dosage is more likely to be among those patients who have a severe renal lesion and poorer prognosis than the group which ceases to excrete protein in the urine.

TABLE 35

STATUS OF PATIENTS WITH NEPHROSIS TREATED WITH STEROIDS

Time	2 years	3 years	4 years
Number of patients	42	32	22
Per cent well	24%	34%	36%
Per cent in remission	24%	12%	9%
Per cent Active	21%	6%	0
Per cent with Chronic Nephritis	12%	23%	0
Per cent Dead	19%	25%	55%

In 1954 - 42 patients have been followed for 2 years
32 patients have been followed for 3 years
22 patients have been followed for 4 years

In Table 35 we have summarized the present status (1954) of children with Nephrosis who have had repeated intensive courses of steroid therapy. In the first column is shown the distribution of 42 patients two years after their initial treatment. If we designate as a favorable group the 24% who are well, plus the 24% in remission (48%), at the end of 3 years, this group, (32 patients followed for 3 years) is 46% and at 4 years 45%, with the patients in remission gradually moving into the group of patients who are well. Conversely, the patients who show continued activity of their disease at the end of two years, may be lumped with the group of patients having chronic nephritis,

GROUP I PROTEIN FREE 22 PTS

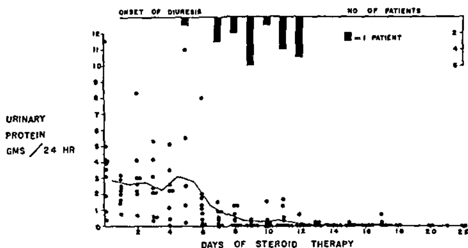


Fig. 94. Effect of steroid therapy on proteinuria and diuresis

GROUP II NOT PROTEIN FREE 13 PTS

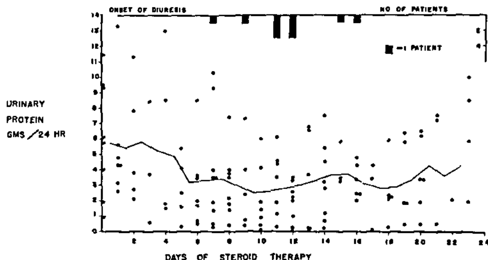


Fig 95. Effect of steroid therapy on proteinuria and diuresis

for at 3 years they have shifted into this category, and at four years have swelled the ranks of the patients dead to 55% of the 22 patients followed for four years.

While this is a pessimistic view, it would appear from our data, that the severity of the lesion in any particular child with Nephrosis is determined early in the disease. Whether steroid therapy alters the ultimate course of the early renal lesion we are unable to say.

Dr McCrory has some observations which have a bearing on early assessment of the magnitude and severity of the renal lesion.

DR WALLACE McCRORY: In the short time available I would like to report briefly on the progress of our studies of proteinuria in children with renal disease. The aim of this study is the clarification of the mechanism of proteinuria in nephrosis and nephritis

If increased glomerular permeability to serum proteins is a major factor responsible for proteinuria, molecular size of the protein particles filtered could be expected to influence their rate of filtration through the glomerulus. One would expect the smallest molecular size proteins (albumins) to pass through the glomerulus more readily than the larger protein molecules (globulins). Clearance rates of the serum protein components should then be highest for small molecular size proteins (albumins) and lowest for large molecular weight proteins (globulins). Hematuria is considered to be a reflection of gross leakage of blood through the glomerulus. If urine protein leaking through glomerular tears allowing passage of blood rather than by filtration the protein composition of urine and serum would be similar. In such a proteinuria clearance values for albumin and globulins would be essentially the same.

Since the amount of protein excreted by the kidney is influenced by glomerular filtration rate and serum protein concentration the quantitative study of proteinuria is valid only when the contribution of these factors is known. Protein excretion will be expressed in terms of protein clearances so that differences in proteinuria between members of the group will not reflect differences in serum protein concentrations. Endogenous creatinine clearances (Hare Method) have been measured as an estimate of glomerular filtration rate. With these data we can indirectly estimate the degree of abnormal permeability of the glomerulus to protein All clearance values have been corrected to one M^2 of surface area.

Paper electrophoresis has been used for the estimation of the albumin and globulin fraction concentrations in serum and urine. Total protein has been determined by the Biuret method Even though the electrophoretically separable fractions of serum and urine proteins are not homogeneous or necessarily similar in composition the globulin fractions contain protein molecules whose average molecular size exceeds that of the relatively homogeneous albumin fraction. (The alpha - 1 - acid glycoprotein, Mol. Wt. 45,000 is an exception) A further recognized limitation of this approach is the fact that it does not provide a means of assessing the important contribution of tubular resorption of filtered protein. Regardless, the presence of significant amounts of protein the urine is evidence of the saturation of functioning tubular mechanisms of protein resorption. We have assumed that tubular resorption affects the concentration

of the individual protein components in the urine of these subjects to a similar degree. Consequently the urinary protein is considered to represent the general composition and the minimal amount filtered through the glomerulus.

The values for the albumin and globulin fraction clearances in the patients are shown in Figure 96. In this qualitative evaluation the globulin fraction clearances are expressed as the percent of the albumin clearance. The albumin clearance is used as the reference standard because this fraction is most homogeneous. If whole serum is being "leaked" into urine the globulin clearances should be similar to the albumin clearance and the ratio would center around one. The data include a group of 18 children with the nephrotic syndrome. Similar data are shown for 6 children observed during the acute stage of glomerulonephritis (AGN), 2 children with chronic nephritis (CN) and 3 children with terminal renal insufficiency (TN) as a result of the nephrotic syndrome. The data for the 18 children with nephrosis reveal that the alpha-1-globulin clearance is the highest of the globulin clearances in comparison to the albumin clearance ($C\alpha\text{-}1/C_{ALB}$ range 0.2-1.15, AVE 0.61). A wide variation in individual values is apparent. This is due in part to the inhomogeneity of the fraction and to difficulties in accurate measurement of low concentrations of alpha-1-globulin by the paper electrophoresis method. The value of the beta-globulin clearance is next highest ($C\beta/C_{ALB}$ range 0.04-0.4, AVE = 0.13) and the alpha-2-globulin and gamma globulin clearances lowest in comparison to C_{ALB} ($C\alpha_2/C_{ALB}$ range .03-.11, AVE = .05; $C\gamma/C_{ALB}$ range .03-.20, AVE = .08). The high value for the alpha-1-globulin clearance could be due in part to the high urinary loss of acid glycoproteins. We have found higher concentrations of the iron binding beta-globulin (Mol. W + 90,000) in urine than in serum. This would contribute to the higher value for the beta-globulin clearances. The finding of lower and different ratios for the major globulin fraction clearance in comparison to C_{ALB} is compatible with the concept that proteinuria in nephrosis is the result of increased glomerular filtration (permeability) of serum protein. To consider another factor we have compared fraction clearance relationships in nephrotic patients who exhibit different degrees of hematuria. The numbers in the bottom of the Graph, I, II, III relate to degrees of hematuria. Group I included children with no or minimal hematuria (up to 10 R.B.C./C HPF), Group II definite microscopic hematuria (11-75 RBC/C HPF) and Group III patients with gross hematuria. The ratios of the globulin fraction clearances to C_{ALB} are however similar. No group separation of these subjects by clearance ratios is apparent. The data for the acute nephritics show a wider variation than the other subjects. The high value for beta-globulin clearance results in large part from hemoglobin which moves with the beta-globulin fraction in urine. In general the values in nephritics show less evidence of selective filtration, but the values for alpha-2 and gamma globulin clearances still differ significantly from the albumin clearance. Though qualitative differences in proteinuria in nephrotic patients were not apparent in relation to the degree of hematuria, a quantitative comparison was more rewarding. Table 36 presents data on the same patients that quantitate their proteinuria. Average figures only for the groups are given in the table. Values for endogenous creatinine clearances (C_{CR}) reveal a lower percent of normal value for C_{CR} in patients with microscopic or gross hematuria. This indicates greater reduction in glomerular filtration in these patients. The proteinuria also was greater as indicated by the higher

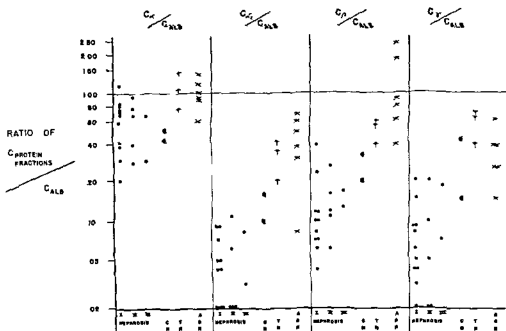


Fig 96 Ratios of protein fraction renal clearance to that of albumin in nephrotic children and in some patients with acute (AGN), chronic (CN) and terminal (TN) nephritis

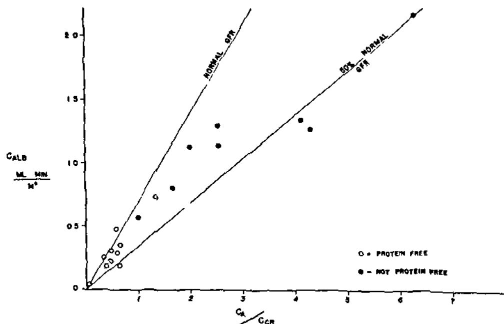


Fig. 97. Relation between proteinuria, albumin and creatinine clearances in nephrotic children

values for albumin clearance in Groups II and III. A measure of the degree of increased permeability of the glomerulus to protein can be obtained by expressing the albumin clearance as a function of the filtration rate. If the creatinine clearance is taken as a measure of the permeability of the glomerulus to water the ratio C_{ALB}/C_{CR} expresses the minimal permeability of the glomerulus to albumin per unit of water filtration. The value for $C_{ALB}/C_{CR} \times 100$ in Group II is 4 times that of Group I, and in Group III, 6 times Group I. The findings of greater permeability to protein and lower filtration rates in patients with microscopic and gross hematuria would suggest the existence

Table 36

RELATIONSHIP BETWEEN PROTEINURIA AND HEMATURIA IN NEPHROSIS

	HEMATURIA RBC's/Cent. HPF		
	0 - 10	10 - 75	GROSS
Group	I	II	III
Number of Patients	10	5	2
% Normal G.F.R *	81.9	74.5	48.3
C_{ALB} ML/Min/M^2S.A.	0 45	1.08	1 3
C_{ALB}/C_{CR}	0 76	2.58	4.2

* C_{CR} ML/MIN/M^2S.A

of greater diffuse glomerular damage quantitatively in these patients rather than a qualitatively different type of renal disease. It is also of interest that similar quantitative differences are apparent in protein clearances in these 18 children with nephrosis if they are separated on the basis of their response to a long (4 week) course of cortisone. Table 37 presents the data with such a group separation. Average albumin clearance values before treatment were 4 times as great in the patients failing to become protein-free Minimal glomerular permeability to albumin was also greater 6 fold) in the patients failing to become protein-free. These differences were found to be statistically significant by the T test A greater depression in glomerular filtration rate was also observed in the latter subjects since values for C_{CR} were lower.

These data would suggest that the patients who become protein-free had milder renal disease The data are graphically shown in Figure 97, the albumin clearance (C_{ALB}) reflects the proteinuria The permeability to protein per unit of filtering surface is shown on the abscissa ($C_A/C_{CR} \times 100$). Two freehand lines are drawn to

Table 37

STATUS OF RENAL DISEASE IN NEPHROSIS

BEFORE CORTISONE IN RELATIONSHIP TO RESPONSE

STATUS AFTER THERAPY	NO PROTEINURIA	$\pm$ PROTEINURIA
Number of Patients	*10*	*8*
C_{ALB} ML/MIN/M^2	0.31	1.21
C_{ALB}/C_{CR}	0.55	3.05
% Normal G.F.R.*	84.6	65.7

*C_{CR} ML/MIN/M^2S.A.

show where values would fall if GFR were normal or 50% normal (C_{CR} data used for GFR). The two groups are clearly distinguishable. It is clear that increasing proteinuria is associated with both increased glomerular permeability to protein per unit of filtering surface and decreasing glomerular filtration rate. Evidence of greater glomerular damage was found in the group who failed to become protein-free with cortisone therapy. Since fraction clearance comparison failed to reveal a qualitative difference these data infer that the patients differ in amount of renal damage rather than in the type. The data suggest that patients showing the best response to cortisone may represent those subjects with mildest renal damage.

CHAIRMAN BARNETT: Here is a clear statement from the group from Philadelphia of their opinion concerning the value of hormone therapy and a suggestion from Dr. McCrory of the means by which prognosis might be indicated early in the disease.

We will go on to Dr Merrill.

DR. ARTHUR MERRILL: I present this as an experimental approach and not my favorite method of treatment. I am sure it is not the best method of treatment for all patients.

Table 38 indicates the treatment which we have used. However, if the patient fails to respond in any way after a period of 3 weeks, we may increase the dose of ACTH to as much as 1 1/4 to 1 1/2 milligrams per pound. Then, as shown in the outline, after the urine has become free of albumin for a period of 2 weeks, we reduce the dose very abruptly, and then somewhat less rapidly with gradually decreasing decrements of dosage. Table 39 shows some factors in response to this form of therapy.

Table 38

TREATMENT

1 mg. per lb. ACTH gel until urine albumin-free for 2 weeks.
Then 1 mg. per lb Q.O.D.
Reduce each dose 3% for 4 weeks.
Reduce each dose 1.5% for 4 weeks.
Reduce each dose 0.5% for 4 weeks.
Give 0.5 mg. per lb. Q 3 days
Stop. Check Albumin Q.D. for 1 month, and twice a week for 1 month. Check with infections for 2 years.
200 mg. Na diet K 6 mg./D. Physical checks. Sulfa 0.5 gm.

Table 39

FACTORS IN RESPONSE

Onset - 9 months to 10 years	NO
Duration (2 weeks to 4 years)	NO
Hematuria (7 patients)	NO
Prior Intermittent Rx (12 patients)	NO
Rapidity Initial Response	NO
NPN Elevation, 6 of 9 resistant	YES
Cholesterol Rapidity of Reduction	YES
Infections	YES

The dose of ACTH is doubled in the presence of an infection instead of omitting it. This seems to help prevent relapse.

Table 40

RELAPSES

CAUSES	NUMBER
Infection	Two have had 2
Reduction in dose	One has had 2
Unknown factors	Four have had 3
	One has had 4
	One has had 6

Table 40 shows the number of relapses. Infection is the most common cause of relapse. Two cases of chicken pox, one of measles, and the rest respiratory infections. Reduction in dosage produces relapse in a fair number of instances, and have occurred after the patients have been taken completely off treatment, one as long as 10 months after the patient had been completely free of albumin and edema.

Table 41 further characterizes the relapses. A trace to 1+ albuminuria does not necessarily mean relapse It is usually abrupt. The patient almost immediately develops a 2 to 4+ albuminuria.

Table 41

RELAPSES

1+ albumin usually means nothing. *Relapse is abrupt,*
2 to 4+ albumin. Anorexia may precede relapse.
Double dose or frequency with relapse or infection until
albumin disappears.
Go back to 1 mg. per lb. daily if albumin fails to decrease
in 4 days or increase after double dose.
Reduce dose rapidly to 25% above pre-relapse.
Then decrease more slowly than before.

Patients have albumin studies every day, but not quantitative. I think the rest of this is pretty obvious.

These represent averages of the way we have treated the patient rather than any fixed, specific way.

Table 42

SUMMARY

Only adult treated well 1 year.
26 children.
1 completely unresponsive.
24 free of edema.
24 free of albumin.
9 off treatment 18 months.
10 still *under treatment.*
No deaths or serious complications.
Usually return to full activity in 2 to 6 weeks.
Brief relapses of albuminuria in 13.
Remain free of edema if followed closely.

Table 42 presents results of treatment to date We have treated only one adult, and we are sure that this is the wrong way to treat most adults because the incidence of complications is so great. Some of the patients have psychotic reactions. *All* develop severe acne and signs of Cushing's syndrome

Twenty-six children have been treated and all but one of these at one time or another have been completely free of albumin and edema.

As of today, 24 are free of edema and have no albuminuria, although 2 of the patients have only been free of albuminuria for 2 weeks following their last relapse. Nine have been off treatment for 18 *months and have remained well. Ten are still* under treatment.

There have been no deaths or serious complications, but these patients have been watched very closely. They have been examined each time they receive ACTH.

Patients are usually able to return to full activity in 2 to 6 weeks and in most instances edema has disappeared in a period of 3 weeks.

In relapses if the dose of ACTH is doubled promptly, the patients do not develop edema. The patients who have had edema in relapses have not had aggressive treatment All but 2 have been rendered free of edema rather easily by an appropriate increase in their dosage.

CHAIRMAN BARNETT: Thank you, Dr. Merrill.

I think, to continue somewhat in the order of people who are doing essentially the same thing, that we might now hear from Dr. Riley and from Dr. Metcoff, and also from Dr. Danowski, whom I didn't mention before. Dr Riley.

DR. CONRAD RILEY: I am more concerned at this time with a method of trying to evaluate what has been going on than I am with the actual results. The accompanying life table form may help in understanding the technique. The question I wanted to answer when we started to get data together was whether or not we are doing anything to the underlying disease.

TABLE 43. Present Status Nephrotic Patients

			STEROID		
	No Rx.	Edema Rx	3-d.	Scheduled Rx 10-d.	28-d.
Excellent	8	5	0	1	0
Good	4	15	7	5	0
Fair	2	4	6	5	6
Poor	0	4	0	3	1
Uremic	0	3	0	0	0
Dead	13	25	1	0	0

Table 43 shows how I tried to answer it at first. This demonstrates how detailed you can be and get nowhere We tried to classify the patients according to present status. I won't define these categories because I am not going to discuss them in detail and we tabulated them according to kind of treatment they have had.

We had a lot of old-timers with no treatment We had a group in whom we used treatment only for recurrence of edema. Then we had a group in whom we used some kind of pre-planned schedule

We had some put on hormone therapy 3 days a week, some 10 days out of each month, and some on prolonged therapy As we looked over our figures of these really well and the others, we didn't have anything very significant. There weren't enough figures, so we next tried to re-combine and get a gross idea of all categories that might mean something

Thus, as seen in Table 44, we limited the categories of treatment to whether they had no treatment, treatment for edema only, and treatment by planned schedule. We reduced the categories of present status to alive and dead

Quite an interesting thing jumped out at one immediately, that among the "no

TABLE 44. Survival vs. Steroid Therapy

	No Rx.	Indicated Rx.	Scheduled Rx.
Alive	14	31	34
Dead	13	25	1
	27	56	35

treatment" group, 13 out of 27 were dead. Out of the "treatment for edema" group, 25 out of 56 were dead. Out of schedule treatment, one out of 34 was dead. That looks wonderful. On the other hand, the follow-up on those in the last treatment group is obviously much shorter.

Table 45

Mean Time of Observation from Onset

	No Rx.	Indicated Rx.	Scheduled Rx.
Alive	67.5 mos.	49 mos.	28.2
Dead	19.8	28 mos.	26 (1 pt.)

So the next thing we did (Table 45) was to make a mean time of death after onset for those that died within the "no treatment" group, and we compared it with the "treatment for edema" group. I must emphasize that the time is calculated from the onset of disease, that is, edema, not the beginning of treatment.

From these figures one sees that those treated by schedule are still alive at least as long as the mean time of death in the other two groups.

So it began to look as if it might mean something; but one can't compare those now alive with those that died very satisfactorily.

So I sought the help of Dr. William Silverman, a very good amateur statistician, and later Dr. John Fertig, a professional statistician. With their help we applied the life table to the problem, as illustrated in Table 46.

This life table, briefly, is a method of trying to evaluate patients according to the duration of their disease. The sample sheet attached has a legend which I hope explains the way patients are distributed on the table. You will see there is a separate table for those patients who received no treatment, those who received treatment for edema only, and those who received treatment on a pre-planned schedule.* In general, com-

*Editor's Note: Dr. Riley further clarified the classification by indicating that all patients treated 6-11 months after onset or later, appear in group 1 until interval treated and then are transfered to group 2. Patients are included in group 3 if indicated hormone therapy was given within one month of start of scheduled therapy. Some of these were non-edematous.

paring the expected mortality (q_x) in the various intervals, it can be seen that there is no very striking difference between those who received no treatment and those who received treatment for edema only. Therefore, it seemed justifiable to pool these two categories as a basis of reference for comparing the effect of treatment on a pre-planned schedule. Using this pooled "probability of dying" and applying it to the total population of the age interval for the patients in the "therapy by schedule" category, we were able to arrive at a figure of an expected death rate (column headed Exp-q_x) and thus the expected number of deaths per interval (Exp-d_x). From this method one calculated that at the end of 65 months the expected number of deaths was 6. The observed number of deaths was one. Calculating the average population per interval we were able to calculate X^2 of the difference expected and observed and found it to be 8 ($P<0.01$)

From this calculation, if the arithmetic is correct, it looks as if treatment on a planned schedule basis is actually prolonging life even though we cannot show that the percentage of complete cures has increased. Our figures are relatively small and it is conceivable that this apparent success is a statistical fluke. I would feel happier if we could get pooled data from all our experiences to which we might apply the same type of technique The attached questionnaire would serve to give the necessary facts to do this kind of analysis. The number of facts asked for here are so few and so simple that it should take only a brief time for any individual to fill out such a questionnaire for a given patient.

DR. BARNETT. I might say at this point that for the last two years we have discussed the desirability of some sort of pooling of data to try to do exactly what Dr. Riley is talking about here. During the past year a group of us in New York have met several times and have prepared a preliminary form which is considerably more detailed than this form that Dr. Riley is now proposing. Before discussing the matter of a coordinated analyses of treatment experiences, Dr. Metcoff will present the observations of his group

DR JACK METCOFF: Part of the problem in the analysis of data relating to the results of therapy in patients with nephrotic syndrome is the lack of statistically significant groups and our inability to assess all pertinent variables.

Dr. John James and I have gone over the patients observed by at least one of us since 1948 to July 1, 1954. We have excluded all patients in whom the onset of the nephrotic syndrome was prior to January 1, 1948

The size of the group is indicated in Table 47.

The patients have been separated into two main categories: Those who received ACTH or cortisone therapy, and those who did not. The untreated "no steroid" group is weighted by patients with either mild disease who went into remission within a few months of onset, or patients who could not be treated because of recurrent complications These patients have been classified further according to whether they were alive, dead or of unknown status. 71% of treated and 53% of untreated patients are alive.

TABLE 46. Life-Table Evaluation of Mortality in Nephrotic Patients

x	1 No Hormone Therapy					2. Hormone Therapy by Indication					3. Hormone Therapy by Schedule						
	lx	nx	dx	wx	qx %	lx	nx	dx	wx	qx %	lx	nx	dx	wx	qx %	qx* exp. %	dx** exp.
0-5	--	64	1	-	3 1	--	48	1	--	4 2	--	30	--	6	0	3.6	0 54
6-11	63	--	2	20	3 8	47	14	3	6	5.9	24	9	1	9	4.2	4 8	1 15
12-17	41	--	3	5	7 8	52	4	3	5	5.9	23	3	--	10	0	6 7	1.31
18-23	33	--	2	4	6 5	48	2	4	5	8.6	16	4	--	9	0	7.7	1.04
24-29	27	--	2	2	7.7	41	2	4	5	10 1	11	3	--	7	0	9 1	0 82
30-35	23	--	1	2	4 5	34	2	5	3	15.0	7	1	--	3	0	10.4	0 62
36-41	20	--	--	2	0	28	1	2	4	7 6	5	1	--	2	0	4.5	0.25
42-47	18	--	1	--	5 6	23	--	--	4	0	4	--	--	--	0	2.6	0.10
48-53	17	--	--	--	0	19	--	1	2	5 6	4	--	--	1	0	2.9	0 10
54-59	17	--	--	3	0	16	--	--	4	0	3	1	--	3	0	0	0
60-65	14	--	--	2	0	12	--	1	3	9 5	1	1	--	--	0	4.3	0 06
66-71	12	--	--	3	0	8	1	--	7	0	2	1	--	1	0	0	0
72-77	9	--	--	1	0	2	--	--	--	0	2	--	--	1	0	0	0
78-83	8	--	--	--	0	2	--	--	--	0	1	--	--	1	0	0	0
84-89	8	--	--	1	0	2	--	1	1	66 5	--	--	--	--	0	11.1	0
90-95	7	--	--	2	0	0	--	--	0	0	--	--	--	--	0	0	0
96+	5	--	2	3	57 1	0	--	--	--	0	--	--	--	--	0	57.1	

Key
x -- 6 months intervals after onset of edema
lx -- individuals living at beginning of interval
nx -- new individuals entering during interval
dx -- individuals dying during interval
wx -- individuals withdrawing during interval (lost to follow-up, currently in interval, transferred to another category).
qx -- death rate per interval -

$$\frac{dx}{lx + 1/2nx - 1/2\,wx}$$

NOTE
At double line, total expected deaths at 65 months 6
Observed deaths at 65 months χ^2 1
of difference (P < 0.01)

*Expected qx was calculated from a pooling of data of 1 and 2.
**Expected dx was calculated on the group using the expected qx.

SUGGESTED QUESTIONAIRE FORM FOR EVALUATION OF HORMONE THERAPY IN THE NEPHROTIC SYNDROME

INDIVIDUAL RESPONSIBLE FOR REPORT ____________________
Name

____
Hospital City

1 Patient's Name ____________________
Last First Middle

2 Date of Birth ____________________
Month Day Year

3 Date of Onset of Edema ____________________
Month Day Year

4. First Came Under Care of Reporting Physician ____________________
Month Day Year

5. Was Patient Seen by Another Physician Previously?
(Check One) ____ Date First Seen, if known ____________
Yes Mo. Day Yr
____
No

6. Present Status: Alive ____________, Dead ________________ (Check One)

7 * Date of Last Contact ____________________
Month Day Year

TREATMENT: This refers only to ACTH, Cortisone, or other comparable Adrenal Steroid

8. No such treatment at any time ____________ (Check if applicable)

9 **Such treatment given for edema only. First given by any physician ____________
Mo. Day Year

10. Such treatment given by predetermined schedule***
First Started ____________
Mo Day Year

* If dead, date of death, if alive, date of last personal observation, correspondence, or telephone contact, whichever is later.

** If patient was treated first for edema, and later by schedule, both No. 10 and No. 9 should be filled out

*** This would include any treatment not given only for edema, such as 3 days a week, 10 days a month, prolonged large dose therapy (longer than 21 days) carried beyond time of diuresis, treatment for chemical signs of relapse without recurrence of edema.

TABLE 47

CHILDREN WITH NEPHROTIC SYNDROME

Jan. 1948 - July 1954

	Therapy		
Patients	Steroid	No Steroid	Total
Alive	85	33	118
Dead	34	28	62
Unknown	7	11	18
	126	72	198

Table 48 presents the same material from another view point. Only patients who received steroids are included in this table. The steroid response in the last course of therapy is related to mortality. No patient is considered twice in this table. Those patients who received only one course of therapy are indicated in the first column; those patients who received two courses, in the second, etc. Of 67 patients who diuresed with the last course of therapy, 4 are dead. Of 31 patients who failed to diurese with the last course, 18 are dead. Of 20 who had an incomplete therapeutic course, 18 are dead. It seems that if a patient responded to therapy his chance of survival to date was greater than if he did not respond or remained untreated.

TABLE 48

CHILDREN WITH NEPHROTIC SYNDROME

Jan. 1948 - June 1954

Steroid Response
Last Course vs Mortality

Patient's Response	No. of Last Course			Total*
	1	2	3-8	
Diuresis	26	19	22	67
(Dead)	(3)	(0)	(1)	(4)
No. Diuresis	9	7	15	31
(Dead)	(6)	(4)	(8)	(18)
Incomplete Course	8	9	3	20
(Dead)	(6)	(6)	(1)	(13)
Total Pts.	43	35	40	118
(Dead)	(15)	(10)	(10)	(35)

* Unknown Status Omitted.

Table 49 attempts to relate therapy and clinical status. Clinical status is defined as clinical remission, dead, and unknown. 71 patients responded to steroids with diuresis on their last course. Of these 43 are in clinical remission, 4 are dead, and the status of 4 in unknown. For those who did not respond with diuresis or had incomplete

courses, 10 are in clinical remission; 31 are dead, and 4 status unknown. Of those patients who had no therapy, 26 are in clinical remission, 28 are dead, and 11 status unknown. It would seem that although fewer untreated than successfully treated patients are alive, proportionately more of the untreated living patients are well.

TABLE 49

CHILDREN WITH THE NEPHROTIC SYNDROME

January 1948 - June 1954

Clinical Status vs Therapy

	No. Patients	Clinical Remission	Active	Dead	Unknown
Steroid Response*	71	43	20	4	4
No Steroid Response*	55	10	10	31	4
No Therapy	72	26	7	28	11
Total	198	79	37	63	19

* Last course of therapy.

It is impossible to draw any firm conclusions from our limited analysis of the data so far I think it is fair to assume that one of two things might account for the status of treated patients Either those patients who responded with diuresis were ones who were destined to get well and hence had the best response, as Drs Rapoport and McCrory have suggested, or those patients who responded to therapy ultimately did better as a result of therapy Our data on serial renal functions in some patients with initially low GFR's who responded to therapy and now are in clinical remission with normal renal functions would favor the latter interpretation in these particular instances.

We are not yet in a position to decide between these two possibilities for most patients, however Analysis of our data along lines suggested by life-table statistics may enable us to do so. However, even with the life table adjustment technique, categorization of patients will be difficult. For example, those patients who we maintain on "Scheduled" treatment are selected with two obvious biases - first, they have remained alive long enough to be treated; secondly they have responded to a full course of hormone therapy with diuresis and are in at least partial remission at the time "Scheduled Therapy" is initiated Obviously such patients are difficult to compare with either an untreated group of patients, or with hormone treated patients who did not respond to full course therapy and therefore were not started on intermittent treatment. Until the statistic is resolved, at least, we will continue to treat patients with steroid hormones

CHAIRMAN BARNETT: Thank you, Jack.

The group from Pittsburgh suggested a new approach to therapy at the last conference. We are eager to know what their results have been during the last year.

Dr. Greenman will present these data.

DR. GREENMAN: I would like to start out by indicating that the prediction that we would continue to have recurrences made by members of last year's conference has been fulfilled On the other hand our therapeutic program is essentially unchanged.

The therapeutic regimen has consisted of bed rest, a diet limited in sodium to two and a half to ten milliequivalents per day, high in potassium, 150 milliequivalents per day, about 3 grams of protein per kilogram of body weight provided with the aid of an electrolyte free protein supplement, and additional iron and vitamins. ACTH, 25 milligrams intramuscularly, every six hours has been given to all patients. In addition *the first twenty-five children received nitrogen mustard, 0.3 milligram per kilogram* of body weight, in single dosage intravenously usually on the third day of therapy.

Dr. Danowski and other members of our group have now treated 41 children, 26 males and 15 females. Of these 33 were less than 5 years old, and 8 between 5 and 15 years of age The duration of illness was less than 2 months in 21 children, 2 to 6 months in 6 others and longer than 6 months in the remaining 14. These 41 children have received a total of 58 courses of the ACTH regimen.

Our experience is like that of other groups insofar as delivery of edema is concerned. Eighteen lost their fluid while on therapy; two gradually delivered their edema; three became edema-free after ACTH was discontinued, and in one edema persisted. The others had had edema delivered by other means.

The effects on the underlying disease state were evaluated by serial physical examinations, urinalyses and blood and serum studies. There are now 24 patients who are completely well; an additional 10 have had an essentially complete remission save that single abnormalities, i.e., trace or 1+ proteinuria or cholesterol values between 300 and 400 milligrams per cent are present. Seven patients are not normal.

Of the 23 patients followed more than 12 to 40 months, 18 are well and 3 have single changes. One has died; one is not well. Thirty per cent have had one or more flare-ups of their illness.

Nine patients have been followed for 7 to 12 months. Six are well and three have had relapses. They have responded to retreatment.

Nine additional patients have been followed between 1 and 4 months. Three are well; 2 have had relapses; 3 are not well and 1 has died. Again the relapses have subsided on retreatment.

Of the 25 patients to whom nitrogen mustard was given, 14 are well. Follow-ups now extend 13 to 40 months. There have been recurrences in 7 from three to twenty-

four months after a favorable response. All relapses have responded to repeat courses of therapy. Four children are not well, 2 were lost after 2 months; 1 died, and one is still ill 19 months after ACTH therapy.

The results in the 16 patients treated without nitrogen mustard are as follows: 8 are well; 4 have single abnormalities; 3 are not well and 1 died of septicemia. Follow-ups now extend for 1 to 27 months with 11 more than 7 months. There have been 5 relapses between 4 and 7 months, again with response to retreatment. There is therefore no evidence in our studies that the inclusion of nitrogen mustard in the therapeutic regimen exerts an additional beneficial effect.

In an attempt to analyze the factors important in patients who will relapse, we can come up only with the following. That 8 of 12 patients with relapses were ill less than 2 months, while 12 of 29 without relapses had been ill for the same interval. At the time of the study there were no differences in blood pressure, heart size, blood non-protein nitrogen, serum albumin and globulin and cholesterol. Urinalyses were not helpful in differentiating the patients. The differences in duration of illness may or may not be significant

In summary therefore, of the 41 children in our unselected series accumulated during a 3 1/2 year period and which has included all instances of the nephrotic syndrome irrespective of the presence or absence of a nephritic component, 39 are still alive. Therapy with rigid sodium restriction, potassium supplementation, a high protein intake, vitamins, iron and ACTH for 28 days produced symptomatic improvement during or following completion of therapy in 57 of 58 courses. Toxic or untoward manifestations were minimal and limited to hyponatremia and water intoxication with convulsions in two One patient developed hypertension and convulsed without electrolyte abnormalities Relapses, responsive to retreatment, have occurred in 30 per cent of the children About 30 per cent of the patients developed upper respiratory infections which responded to simple therapy. Two of them were serious. Analysis of chemical or laboratory features preceding or predisposing to an exacerbation reveals a somewhat shorter clinical course prior to institution of therapy in those who ultimately develop a flare-up. At any particular moment 85 per cent of our children are in a complete or virtually complete remission, though about one-third of these may have possible abnormalities which are difficult to evaluate. These consist of trace to 1+ albuminuria and serum cholesterol levels between 300 and 400 milligrams per cent. We feel therefore that this therapeutic regimen presents some measure of control of the underlying disease state, beyond the mere symptomatic relief which has been the usual goal in the past. We do not know that all of the components of the regimen are indispensible.

CHAIRMAN BARNETT: Next we would like to hear the experience of the group working with Dr. Lange who with Dr. Kramer, I think, were probably the first to recommend long term treatment. Dr. Strang will present their experiences.

DR. STRANG: I agree that it is difficult to evaluate these groups statistically. First, our number of cases is small and second, because we have been seeking a better method to prolong remissions of the nephrotic syndrome, we have used several different schedules of intermittent maintenance therapy.

In 1951 Dr. Lange suggested that remissions of the nephrotic syndrome might be prolonged if ACTH or Cortisone was given after diuresis had occurred. We knew that it was impractical to give high doses of ACTH or Cortisone daily for a long period of time because of the undesirable side effects. Experience soon taught us that if low doses were given daily, the patients relapsed while on maintenance therapy. The idea was conceived then to give patients high doses of ACTH (100 mg. daily) or Cortisone (400 mg. daily) during regularly repeated, short periods of time. Accordingly, it was planned to give ACTH or Cortisone for three consecutive days followed by four days of no therapy. One week of therapy as described was designated as one maintenance course.

In our original work ACTH was used for maintenance therapy. Five days after diuresis had been induced by 7-10 days of ACTH, patients were given ACTH 100 mg. daily for three successive days. For four days thereafter no ACTH was given.

The first group of patients was small, (Table 50) consisting of five children and one adult. The average number of maintenance couses was five, the range being 3 to 8 The number of relapses which occurred was three, and the time interval between the end of therapy and the occurrence of relapse averaged 7 months (range: 4 to 12 months). At the present time these particular patients have been observed from 23 to 37 months.

TABLE 50

Maintenance Therapy and Relapses

	"Short-term" Maintenance Therapy	"Long-term" Maintenance Therapy
Number of Patients	6	18
Number of Maintenance Courses	3-8	6-30
Average Number of Maintenance Courses	5	Relapsing Cases: 12 Remissions sustained: 20
Number of Relapses during Therapy	0	0
Number of Relapses after Discontinuation of Therapy	3	5
Time Interval between End of Therapy and Relapse	4-12 months	4-8 months
Period of Observation	23-37 months	6-36 months

It became apparent that even though this group was small the number of relapses was significant, but it seemed important that no relapses occurred while these patients were receiving therapy. In each case relapse developed after the discontinuation of therapy. The question which came to us was therefore, would prolonging maintenance therapy decrease the incidence of relapses?

When we considered prolonged maintenance therapy with ACTH, several objections were raised. Our first group had been hospitalized during therapy. Prolonging therapy would then necessitate either too long a hospital stay or the inconvenience of arranging for intramuscular injections at home. The many intramuscular injections were a consideration, especially, because the majority of our patients were children. Oral Cortisone (400 mg daily) therefore seemed more practical. The patients would be permitted the comforts of home and no injections and therapy could be supervised adequately by clinic visits.

Eighteen patients, 16 new patients and two from the original group, have received this type of therapy The number of maintenance courses has ranged from 6 to 30, the longer terms of therapy being given to patients who have started therapy during the past year.

Analysis of case material on August 1, 1954 disclosed that five relapses have occurred. These statistics have not changed during the past two months. Again, no relapses developed while these patients were on therapy. The time interval between the end of therapy and relapses has averaged 5 6 months, the range being 4 to 8 months

When this group was divided into relapsing cases and "remission-sustained" cases, we found that in the relapsing group the number of courses of maintenance therapy has averaged 12, the range being 6 to 20. Patients who have had no exacerbations have received 12 to 30 maintenance courses, the average being 21. The period of observation of these cases has been 6 to 36 months (average· 18 months)

In this group we have had one death. This particular patient was treated with ACTH in the hospital Diuresis was induced and oral Cortisone maintenance therapy was started The child then returned to his home in another city and therapy was supervised there After many weeks oral Cortisone maintenance therapy was discontinued and, according to the report received by us, five months later the child developed a very severe infection which lasted 1-2 weeks and was brought under control with difficulty. Shortly thereafter massive proteinuria developed and edema became obvious. Before steroid therapy was restarted, the child expired suddenly one morning without warning. Because an autopsy was not performed, the cause of death is undetermined.

At the present time the other seventeen patients are free of edema and clinically well They have either no proteinuria or only a mild degree

I would like to reemphasize that in our group of cases no relapses have occurred during therapy All relapses have developed after the discontinuation of therapy, and these relapses have been preceded by one or several episodes of acute infection, most frequently pharyngotonsillar or respiratory

Because of our experiences, we believe now that long-term maintenance therapy does prolong remissions We are still observing relapses after therapy has been discontinued and, therefore, the question to us is, just how long should maintenance therapy be given?

CHAIRMAN BARNETT: Thank you.

We are getting various shades of opinion, I think, about the value of the long-term therapy.

I think Dr. Kramer was the first one, at least to my knowledge, who treated non-edematous children with hormones, and Dr. Kramer, we would be interested in knowing your present ideas.

DR. KRAMER: Dr Metcoff said that his 198 cases were too few for statistical analysis and that no conclusions could be drawn. I can only echo that sentiment based upon an analysis of our 17 patients. Obviously, any conclusions which I may draw must be considered very, very, tentative. However, I could not help forming some impressions even from a study of this small group.

We selected for study only cases that had received one or more courses of either ACTH or Cortisone. The patients were all treated in the hospital for their edema and were discharged when edema free. They received from 60 to 125 mg. of ACTH in 4 divided doses daily for a period varying from 10 days to 4 weeks. If diuresis set in before the 10 day period was up the dose was rapidly decreased and further hormone therapy was withheld until diuresis was complete The following changes were considered an indication that diuresis was about to set in and the dose of ACTH was then either decreased or omitted completely: a decrease in albuminuria, a rise in serum albumin level, a drop in the sedimentation rate and a decrease in cholesterol concentration. After diuresis ceased hormone therapy was resumed and continued until the blood chemical findings had returned to normal. A few cases received twenty consecutive days of hormone therapy without regard to diuresis. Occasionally, the gamma globulin level was still low when treatment was stopped. Patients showing no edema and normal chemical findings in the blood were discharged even though albuminuria persisted and treatment was resumed only with the reappearance of edema. More recently, we have followed Dr. Lange's suggestions and administered Cortisone intermittently, i.e., 3 days for treatment each week and 4 days without treatment. No attempt was made to control salt intake except that vegetables were cooked in the usual manner and no additional salt was served.

We had a total of 18 cases of which we were able to follow 17. These were divided equally between males and females. Their ages varied between 16 months and 4 1/2 years at the time of onset of their disease They received from 1 to 6 courses of ACTH and in cases receiving Cortisone they were given from 1 to 3 courses. At the present time, of the 17 cases followed, 11 are alive and 6 are known to be dead. Of these, 5 died a renal death and one died of sepsis, giving a mortality of about 35%. Eight are known to be clinically well, but 5 of these still show variable amounts of albumin in the urine from zero to 3+. Of the 8 cases 1 has been well for 4 months, 1 for 1 year, 1 for 1 1/2 years, 2 for 2 years, 2 for 3 years, and 1 for 4 years. Of the three patients who received maintenance therapy 1 received 200 mgs. of Cortisone a day for 3 days in each week and the other only 5 mgs. a day for 4 days in each week. One of these patients is dead. Of the 2 living patients 1 has been well for 1 year and the other for 3 years. We attempted to correlate certain phenomena such as hematuria, azotemia and hypertension, with mortality. A persistence of any one of these must be considered

a grave sign, particularly persistent azotemia and, or, hypertension.

Children who are given ACTH in what we considered as a therapeutic dose and failed to develop diuresis, but showed a persistent elevation of NPN and hypertension either during the first course of therapy or during the second course and if these symptoms persisted after therapy was discontinued, usually did very poorly, so that we feel that this abnormal response to therapy may be an indication of structural irreversible damage to the kidney and is therefore an important prognostic sign

This would indicate the need for a further subdivision of patients presenting the nephrotic syndrome into those patients who present the uncomplicated syndrome of lipoid nephrosis and those that have persistent azotemia, hypertension and hematuria. With prolonged illness, the likelihood of unfavorable complications increases, while the chances of survival diminish A statistical evaluation of these superficial impressions is certainly indicated.

CHAIRMAN BARNETT· Thank you, Dr. Kramer.

I have left the Cleveland group to the end because they are the least likely ones that have to leave early

Before they start, and just to change the subject a little bit, I would like to show one slide concerning infections in Nephrotic children and the problem of continuous prophylactic antibiotics

During this past winter we have separated our children into two groups. We treated one-half of them continuously alternating at monthly intervals between 10 milligrams per kilo of Terramycin* and 300,000 units a day of Bicillin**

The other group were treated with the same antibiotics but only for indication However, they were treated more liberally than we recommend for most other children

We counted the number of months in which a child had an infection with fever of over 101, and then calculated the per cent of months in the observation that a child had such infections.

In Figure 98 the black columns represent data on children who received continuous prophylactic treatment The stippled columns represent data on children who were treated intermittently for indication. Although this is not a large group, there appears to be a striking difference between the two groups.

The groups are so small, and this is so hard to evaluate, that during the current year we have reversed our groups. The children who last year got antibiotics continuously this year are getting it intermittently We hope this device will eliminate some of the bias If, at the end of this year, it comes out the same way, we will tend to believe that one can reduce the incidence of infections with fever by continuous administration of antibiotics and we will then probably recommend treating all children with continous prophylactic antibiotics.

* Kindly supplied by Pfizer & Co
** Kindly supplied by Pfizer & Co

Now, may we go on with the last three discussions of therapy and then perhaps have a short intermission. After the intermission I should like to ask Dr. Guild if she would open the discussion because I think we would all be interested in her judgment about these reports and her own experiences.

Dr. Spector, would you begin?

DR. SAMUEL SPECTOR: Much of the data we have to present has already been presented by other members, primarily the Philadelphia group, and also the Boston group. I will try to emphasize some of the differences we observed.

Our data concerns short-term therapy. ACTH or Cortisone was administered for ten to twelve days and repeated only when edema became apparent and the child showed some difficulty I think the latter depended a good deal on the judgment of the individual treating the child.

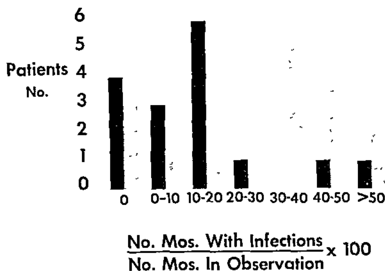
In Table 51, it is noted that the incidence of diuresis was the same whether ACTH was administered I.M. or I.V. or if Cortisone was used. However, in the group of children who eventually died of this disease, diuresis occurred in 54% of the cases in contrast to 81% of the entire group. One might infer that the children who died had a chronic stage of the disease which was responsible for the failure to respond. Yet, in most of these patients failure of diuresis occurred at a time when definite indication of progressive renal disease was not evident. When a child did have evidence of fixed renal impairment, hormone therapy was no longer employed. Of 13 patients who failed to respond twice during their course of hormone treatments, 11 have died to date of renal failure. Thus, it appears that failure to diurese is a poor prognostic sign.

Length of remission appeared to be longer following ACTH therapy (4.2 months) than after Cortisone therapy (2.6 months). The data were not sufficient to be statistically significant.

TABLE 51

ACTH or Cortisone: 189 Courses
(10/49-4/54)

	ACTH I.M.	ACTH I.V.	Cortisone
No. Courses	117	33	39
Incidence of Diuresis	85%	76%	74%
Overall	81% (54% in group who died)		
Diuresis During Treatment	69%	71%	80%
Length of Remission	4.2 mo.		2.6 mo.



$t^2 = 6.55$

$p < .01$

Fig 98 Effect of continuous prophylactic antibiotic therapy on incidence of severe infections in nephrotic children (Continuous antibiotic as black columns, intermittent antibiotic, stippled)

Complications as listed in Table 52 proved infrequent and usually reversible. However, several of them should be emphasized. We had 1 child who at the end of a short term course of ACTH (10th day) suddenly developed high fever, severe abdominal pain and a mass could be palpated in the left flank. Although he recovered from this acute insult, he succumbed 8 months later and proved to have a thrombosis of the left renal vein and contracted left kidney. Whether this thrombosis was induced by ACTH therapy is difficult to determine, but it raises a problem in regard to the safety of long-term hormone therapy with either ACTH or Cortisone.

TABLE 52

Complications (189 Courses)

Transient Hypertension	
(Diastolic 90) .	22
Hyponatremia	3
Hypokalemia	2
Hypocalcemia·	2
Convulsions.	6
Renal Vein Thrombosis.	1

Another interesting complication from viewpoint of prognosis was convulsions. We had 6 convulsions occurring in 5 patients. Three of these children had hypocalcemia. However, in 2, there was no explanation, no evidence of hypertension or renal impairment. All 5 children have died.

TABLE 53

RESULTS

				Number 64	%
Dead				17	26.5
Chronic Renal Disease				1	1.5
Active				15	23.
Remission				19	30.
	> 4m·6,	> 1y:5,	> 2y:8.		
Probably Cured				12	19.
	> 6m·3,	> 1y:4,	> 2y.5.		

Table 53 lists our results in the 64 children we have treated. The shortest time any of these patients has been followed was 14 months and more than 50% of the group have been followed for more than 2 years. Seventeen or 26% have died. One has chronic renal disease which has been stationary for more than 2 years. Fifteen or 23% are still active, but have no evidence of fixed renal impairment Nineteen or 30% are in remission which, as defined by us, is freedom of edema with minor degree of albuminuria and no evidence of chronic renal disease of more than 4 months' duration. Thirteen of these patients have been in remission for more than 1 year and, from past experience, we feel that this group will probably progress to cure. Twelve or 19% have been considered as probably cured. They are symptomatically and chemically normal. It is our estimate that some of the active patients will eventually develop chronic disease so that the eventual mortality in this group will rise from the present 26% to approximately 35%, a figure closely approximating our results in a previous study performed before hormone therapy but after the advent of antibiotics.

It is interesting to note from Table 54 that with this regimen of therapy the majority of our patients needed 2 or less courses.

TABLE 54

COURSES

32% - 1 Course (12 days hospitalization)

53% - 2 Courses (3 wks. hospitalization)

69% - 3 Courses (1 1/4 mos. hospitalization)

80% - 4 Courses (1 2/3 mos. hospitalization)

In reviewing our deaths, 14 of the 17 died in renal failure. One child died following renal vein thrombosis. One child had an E. Coli sepsis complicated by an acute necrotizing pancreatitis.

Ten of the children had no recognized evidence of chronic disease at onset of therapy. Eight of these were treated within 2 months of onset and despite 3 - 8 courses, died in 8 - 24 months of renal failure. These findings seems to indicate that the short-term treatment with either ACTH or Cortisone does not prevent progression of the renal lesion. It does decrease morbidity and period of hospitalization.

CHAIRMAN BARNETT: Thank you, Dr. Spector.

We have here another impression that repeated failure to respond to ACTH might have a prognostic significance, indicating a poor prognosis.

We now will hear from two of the people, I think, who have had recent experience with the use of nitrogen mustard, and we will start with Dr. Taylor.

DR. ROBERT TAYLOR: There are several things that I would like to say very briefly before beginning this discussion. First, I am not particularly interested in pediatrics, but rather internal medicine, and hence the patients we have are almost all adults. Our approach to the problem assumes that all have chronic glomerulonephritis. Nephrosis is something I don't understand.

All patients have been followed for at least three years subsequent to their treatment, and most of them for five years.

Nitrogen mustard was given at the rate of a tenth of a milligram per kilo on each of four successive days. As Dr. Chasis and Dr. Goldring described in 1948 or 1949, the nitrogen mustard caused transient diminution of proteinuria but it bounces back after treatment, and doesn't become normal for weeks or months later

We have studied twenty-two patients for three to five years after treatment. They are in three groups, those who recovered, those who are unchanged, and those who died. Nine patients have apparently recovered completely without any residue of their disease (Table 55). Their ages range from two to forty-eight years of age.

TABLE 55

PATIENTS RECOVERED AFTER HN_2

	Age	Sex	Duration Mos.	Hematuria Cells/12 hrs.	Urea Clearance % of normal
	40	F	2	0 5	45
	48	M	3	0.9	55
	43	M	6	19.0	97
	21	F	2	1.5	47
	3	M	6	0 6	84
	5	M	2	0.3	50
	3	F	6	0 8	100
	5	M	8	0.4	30
	2	M	9	Gross	45
Ave.	19		5	3.0	71

The number of months the disease had been present before treatment was given, from two to nine, is indicated in Column 3. Symptoms were present for an average of five months before treatment.

Column 4 shows the amount of hematuria by Addis counts. It ranges from one patient with gross hematuria to normal which is less than half a million red cells in twelve hours. The average was 3.0 million cells. Urea clearance values (column 4) averaged 71 per cent of normal before treatment, and ranged from 30 to 100 per cent of normal. Average age was 19 years.

Two children, two and three years of age respectively, had between them five relapses, but both have been well for three years since the last relapse. Each recurrence was successfully treated with nitrogen mustard.

TABLE 56

DISEASE UNCHANGED AFTER HN_2

	Age	Sex	Duration Mos.	Hematuria Cells/12 hrs.	Urea Clearance % of Normal
	58	M	3	16.0	65
	14	M	5	24.0	40
	26	M	3	27.0	115
	17	F	11	4.4	30
	4	F	3	12.0	62
	21	M	6	20.0	80
	41	M	1	1.8	85
Ave.	26		4.6	15.2	69

In the patients of Table 56, the disease was uninfluenced by nitrogen mustard. Their average age was 26, but ranged from four to 58 years. They had symptoms about the same length of time as the previous group.

The only difference that we can find between those who did well and these is that more of the latter group had significant hematuria. The lowest in this group is 1.8 million per twelve hours, the average 15 million. Urea clearances were roughly the same, the average 69 as compared to 71.

The last group of patients are now dead (Table 57). All had rather far advanced renal disease. Azotemia, with urea clearance of 10 per cent was present in one. A youngster of 8 years had a normal clearance, minimal hematuria and his disease had been present for ten months before he died. Why he died, we don't know for sure, but he did not respond.

TABLE 57

PATIENTS EXPIRED AFTER HN_2

	Age	Sex	Duration Mos.	Hematuria Cells/ 1 hrs.	Urea Clearance % of Normal
	25	M	18	18.0	10
	54	M	12	50.0	35
	57	M	17	10.0	45
	69	F	7	4.8	48
	3	M	24	4.0	27
	8	M	10	0.8	85
Ave.	36		14.7	16.3	42

Why some of these patients respond and why some don't, we do not know The only difference we can see is that the patients who did well (Table 58) have slightly less hematuria and slightly better renal function. Thank you.

TABLE 58

SUMMARY OF THREE GROUPS OF PATIENTS

	Ave. Age	Duration	Hematuria	Clearance
Recovered	19	5mos.	3.0 mil.	71%
Unchanged	26	4.6 mos.	15.2 mil.	69%
Expired	36	14.7 mos.	16.3 mil	42%

CHAIRMAN BARNETT· Thank you, Dr Taylor.

The next speaker will be Dr. Startzman.

DR. VIOLA STARTZMAN: I wish to present a small group of eight patients who have had a combined course of ACTH and nitrogen mustard.

It has been a set course of ten days of ACTH, 10 milligrams every six hours intramuscularly, for the ten-day period. On the sixth, seventh, eighth and ninth day they received intravenous nitrogen mustard, calculated at one-tenth gram per kilogram of body weight.

The patients have been followed until November 1 of this year.

All of these patients had therapy of one type or another but relapsed preceding this combined course

7 patients are in remission following the last course of combined therapy for 1-22 months; 4 of these for longer than 9 months. 1 patient, a child recently treated, failed to respond to therapy.

CHAIRMAN BARNETT: Thank you Dr. Startzman.

I think we would be very interested, Dr. Guild, if you would be willing to tell us what you are thinking at present about the use of the hormones.

I would be interested also in your present thoughts about treatment and importance of infection in this disease. Dr. Guild.

DR. HARRIET GUILD: I have a few illustrations which serve to illustrate some of the points that have already been made today.

Since the cortisone gospel has spread, the patients are getting to us earlier and we are now having more of an opportunity to follow a group from the start of the disease. While many of the out-of-town patients still return to their own physicians, who usually cooperate well in continuing treatment according to plan, more and more of the local patients are being left in our hands for treatment throughout the course of their disease.

A year ago I made a rough summary of the patients treated with cortisone up to that time, covering a period of three years These were divided into three groups: (1) those in whom the disease started as a "pure nephrosis" and has remained such throughout the period of observation; (2) those in whom it started with purely "nephrotic" signs but in whom a "nephritic" episode or episodes with hematuria, nitrogen retention and elevated blood pressure have supervened; and (3) those in whom the "nephritic" component has been conspicuously present from the start. While this is rather loose terminology, I am sure you know what I mean. One patient, in remission when last seen, was accidentally omitted from the first group; and one, now dead, was omitted also from the third group. Thus there should have been thirty patients in all.

In Figure 99 are shown the patients in these groups. A four plus (++++) response to treatment indicates complete disappearance of all clinical signs and chemical evidence of the disease, including albuminuria, three plus (+++) indicates disappearance of everything except the albuminuria, two plus (++) indicates moderate improvement with edema stablized at a lower level but without complete return to normal; and one plus (+) just very slight improvement of brief duration. Each course of treatment is recorded separately for each patient, although, for purposes of condensation, the duration of treatment is not indicated. Where an arrow follows the recorded response (for example `+++ →), cortisone has been continued in maintenance dosage after the maximal response has occurred.

	Age at Onset	Duration At First Visit	Total To Date	Courses of Cortisone With Degree of Response 1	2	3	4	5	6	Present Status
1	3 yrs. 9 mo.	5 mos.	3 yrs	++++	Subsequent slight albuminuria					Well
2	2½ yrs.	5 mos.	4 yrs.	++++						Well except for occ. alb.
3	2½ yrs.	9 mos.	4 yrs.	0	+++	++	→			Albuminuria and occ. slight edema recently.
4	2 yrs.	1 mo.	2 yrs.	++++	++++	++++	++++	++++	++++	Well
5	2½ yrs.	6 mos.	1½ yr.	++++	++++	→				In remission
6	1 yr.	2 wks.	1 yr.	++++	++++	++++	→			In remission
7	2 yrs.	1 wk.	1 yr.	++++	++++	→				In remission
8	1 yr.	2 wks.	9 mos.	++++	++	→				Disease active but general condition good.
9	2½ yrs.	1 mo.	4 mos.	++++	→					In remission

10	4 yrs.	2 mos.	d.1½ yrs.	++++	+++	++				Dead
11	5 yrs.	7 mos.	3½ yrs.	++++	++	++	+++			C.V.A. 2 yrs ago with seque- [illegible]
12	6 yrs.	6 wks.	3 yrs	[illegible]						[illegible]
13	1 yr.	2 wks.	1½ yr	[illegible]						[illegible]
14	2 yr. 10 mo.	4 mos.	1 yr. 4 mos	[illegible]						[illegible]
15	16 mos.	2 yrs.	3 yrs	[illegible]						[illegible]

	Age at Onset	Duration At first Visit	Total To Date	Courses of Cortisone With Degree of Response 1	2	3	4	5	Present Status
16	2 yrs.	10 mos.	4 yrs. 9 mos.	+++	+++	+++	+++	Just Begun	Nephrotic phase subsided. Nephritis progressing since #4.
17	17 mos.	4 wks.	(d) 2½ yrs.	+	Duration 1½ yr. at start of therapy				Dead
18	20 mos.	1 mo.	4 yrs. 9 mos.	++					Nephrotic phase subsided. Kidney function still improving.
19	3½ yrs.	6 mos.	(d) 2 yrs.	+	++	+			Dead
20	22 mos.	6 wks.	(d) 6 mos.	++	++				Accidental death (shock following paracentesis.)
21	14 mos.	1 yr.	3 yrs.	++	++	++	++	→	Both components smouldering but maintaining "status quo"
22	2 yrs.	3 mos.	(d) 8 mos.	+					Dead
23	4 yrs.	2 wks.	2 yrs.	+++	+++	+++	→		Recent flare-up, complicated by erysipeloid infection. Subsiding.
24	2 yrs.	3 wks.	1½ yrs.	++++	ACTH ++	Just Begun			ACTH in Puerto Rico. Kidney function good but hematuria.
*25	4 yrs.	4 yrs	(d) 4½ yrs.	0					Dead
*26	7 yrs.	3 wks.	(d) 6 mos.	0					Dead
*27	13 yrs.	3 wks.	(d) 4 mos.	0					Dead
*28	10 yrs.	1 yr.	2 yrs.	0					Temporarily static with impaired function

Fig. 99. Cortisone treated patients, 1950-1953

A. Uncomplicated nephrosis - Patients No 14 and No. 15 not observed during initial nephrotic phase (history only).

B Nephrosis later showing nephritic signs

C Nephrosis with concomitant nephritic complement

* Cortisone given to these without expectation of success

No. 25 - renal disease too far advanced.
Nos. 26, 27 - nephritic predominated and was rapidly progressing from onset.
No 28 - blood pressure too high for adequate dosage.

In the first group (A), patients 1, 2, 4, 8 and 9, are now completely well or in remission, No's 3, 5 and 6 are now in remission, on cortisone, which has been given continuously for over a year. The maintenance dosage has averaged 50 milligrams a day. Recently, however, we have been using larger initial dosage and have given more than 50 milligrams daily for maintenance if more seemed indicated.

In the second group (B), the sections underlined represent the periods during which "nephritic" signs were present. The first child (No. 10) had a complete response to the first course of cortisone, relapsing two months after it was withdrawn There was a long delay before treatment could be resumed and we wondered whether that played a part in his subsequent failure to get well. We later adopted the policy of giving treatment for a longer period of time and of stepping in more quickly with resumption of treatment when signs of relapse appeared. This child progressed to a chronic nephritis and died Thus far his is the only death in this group.

The third child (No. 12), whose course will be shown in more detail, after going through a "nephritic" episode, entered a purely "nephrotic" state with gradual disappearance of all signs of the disease except for the albumin in the urine. Because of the albuminuria, maintenance therapy was continued, after the fifth course of treatment, for another year Six months before this slide was made, cortisone was withdrawn for a short time but was then resumed because of a consequent increase in chemical signs of the disease. It has since been continued for another eighteen months and the child is now completely well except for a trace to one plus albumin

In the fourth child of this group (No 13), "nephritic" signs were of relatively brief duration They seemed to be suppressed completely by cortisone therapy and the child is well now except for a mild albuminuria. He is still on therapy

The sixth one (No 15), had the disease for two years before seen by us. He has shown a most remarkable, really unexpected improvement in kidney function on cortisone therapy. He is doing well on maintenance dosage a year and a half after cortisone was started

In the third group (C), the picture is quite different These are the patients in whom the "nephritic" component has been prominent from the very start. At the time this illustration was made a year ago, already seven of the thirteen had died and another has since succumbed

These patients all had hematuria, nitrogen retention and elevated blood pressure when we first collected them and these findings were supposed to have been present from the onset of the disease They progressed to a chronic nephritis and death from renal failure, with one exception.

As far as the other patients in this group are concerned, it is not worthwhile to discuss them individually The chart illustrates the difference in response in the majority of patients in whom "nephritic" signs are prominent from the start. But it also shows that one need not give up hope for them and also need not dodge cortisone therapy just because kidney damage (with even a considerable degree of impairment of renal function) is evident when the patient first comes under observation

I shall now illustrate the various types of response described within the different groups. The line across the center of the following figures represents the normal level for each of the chemical findings recorded. In the columns for serum proteins, the solid upper part of the column is the albumin fraction; the lower is the globulin The symbols for the other elements are self-explanatory.

Figure 100 illustrates the course of a child (No. 4) who had six complete remissions on interrupted therapy. Since the chart is not up to date, only the first five courses of cortisone are shown. Every remission lasted for two months. In the first relapse, treatment was not resumed until edema reappeared; in all of the others, it was started as soon as there was a significant degree of albuminuria before edema had time to appear This child has been completely well now for a year without cortisone. As has been pointed out already today, she is the type of patient who probably would have recovered anyway. One feels, however, that by keeping her edema-free and in an artificial state of remission during the active stage of the disease, one has helped her to ride through her disease more comfortably and safely, and has also eased the situation for the whole family.

Figure 101 illustrates the course of one of the patients (No. 12) in the second group who started with a "pure nephrosis" and who had responded well to the initial course of cortisone therapy. The chart begins with the first relapse which occurred with a series of infections. By now there was a "nephritic" component. After chronically infected tonsils were removed, she was given two short courses of treatment, comparable in duration and dosage to the one that induced the initial remission, but with only partial response. The parents removed her from the hospital against advice before more intensive treatment could be given. She coasted through the summer fairly well, but returned in the fall with a severe exacerbation in the "nephritic" component. This time the parents agreed to leave her until we were satisfied that enough cortisone had been given. After two months of therapy the "nephritic" signs subsided; within another month edema had disappeared, "nephrotic" chemistries were approaching a normal level; and a few weeks later she was in complete remission except for a mild albuminuria. A small maintenance dose of 25 mgm. of cortisone daily was continued for a year, when it was withdrawn for a period of two weeks. Following withdrawal there was an increase in the albuminuria and a slight rise in non-protein-nitrogen. Cortisone was, therefore, resumed. The child has remained well throughout this period, showing only a trace to one plus albumin, and we are now in the process of weaning her from the cortisone again

Figure 102 demonstrates the effect of infection in precipitating relapses. This child (No. 13) who had been in remission for two months following his first course of cortisone therapy had therapy resumed because of the reappearance of albuminuria, when an acute infection precipitated a complete relapse. Four infections in a row kept precipitating relapses just as he was trying to go into remission. Following the fourth infection, a year passed before another remission was achieved and during this period there was a transient "nephritic" episode of about two months' duration. Still on maintenance therapy this little boy has been without edema since last February and in chemical remission except for a low-grade albuminuria since April.

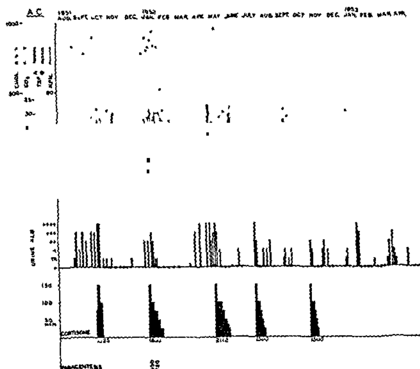


Fig 100 Example of repeated therapy for recurrent albuminuria (Case No. 4).

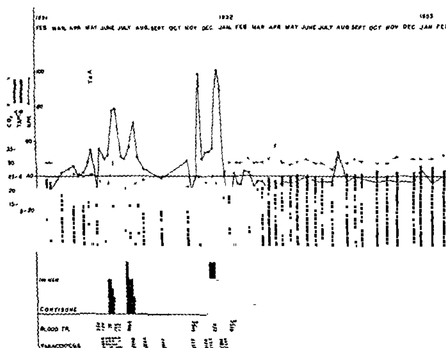


Fig. 101. Example of continuous cortisone therapy with improvement and persistent remission (Case No 12).

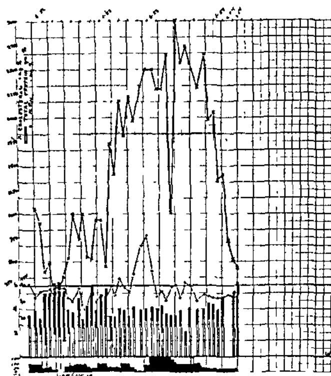


Fig. 102. Effect of infection in precipitating relapses Complete course of Case No. 13 (Group B) from onset in May 1952 to April 1954.

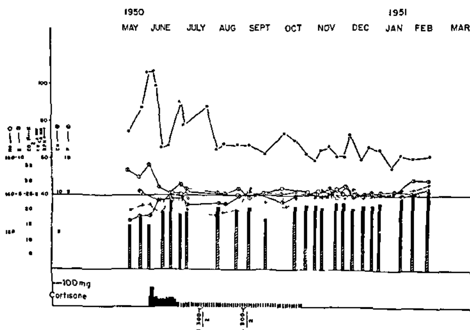


Fig. 103. Example of improvement in renal disease following cortisone therapy (Case No. 18)

Figure 103 illustrates the course of the child previously referred to in the third group, who had gross hematuria and a non-protein nitrogen of 90 mg.% at onset. While the non-protein nitrogen had gradually settled down to an average level of 60 mg.%, there had been slowly progressive renal insufficiency during the 18 months before cortisone became available. The chart begins with a sudden exacerbation in the renal disease when there was an abrupt rise in the non-protein nitrogen to 100 mg.% and a rise in serum phosphorus to 10 mg.% The kidney could not longer concentrate the urine and the 2-hour phthalein excretion was less than 10%. Furthermore the blood pressure was up to 160/100 While the child appeared to be heading into the terminal stage and it did not seem likely that cortisone could help, the abruptness of the immediate exacerbation made it seem worthwhile to give it a trial. Treatment was started cautiously by the intramuscular route and to our surprise the non-protein nitrogen dropped back within a few weeks to an average level of 60 mg.%, the electrolyte picture gradually returned to normal and, over a period of several months, even the proteins reached normal for the first time, edema disappeared and the blood pressure returned to normal. After five months of treatment, the cortisone was withdrawn and steady improvement in kidney function has continued with no relapse over a period of three more years. The child is still in excellent condition, with a non-protein nitrogen that hovers between 40 and 50 mg.% and a 2-hour phthalein excretion of 65% The capacity of the kidney to concentrate the urine is still impaired and there is still a mild degree of albuminuria with a few casts from time to time in the urinary sediment, but he is free of external signs of his disease and his blood pressure remains normal. While this child has had ideal home care and much of the improvement has been accomplished on his own, one cannot escape the feeling that it was the cortisone that turned the tide at the critical moment, by suppressing what would probably have been a fatal exacerbation

In conclusion, I think we can say that cortisone produces remissions most easily in the purely "nephrotic" group of patients in whom there is no evidence of glomerular damage While these are the ones who would probably have recovered anyway and while the cortisone probably does not shorten the course of the disease, they ride through the disease more comfortably with fewer complications, and it certainly seems desirable to keep them in remission, even though it is an artificial one, as long as it is possible

In the group of patients in whom "nephritic" episodes occur, spontaneously or precipitated by infection, one has the feeling that cortisone often helps to suppress the "nephritic" process, thus favoring chances for recovery. While some of these undoubtedly would recover by themselves, there are others in whom the balance may swing one way or the other and in whom cortisone may give just enough of a shove in the right direction to push them back over the fence

In the last group of patients, in whom subacute or chronic nephritis is progressing, I doubt whether, even with cortisone therapy, we can expect complete reversal. By suppressing the disease, however, and keeping the lid on for a reasonable period of time, it has in some instances prolonged life and has at least permitted the disease to become quiescent Only time will tell whether the ultimate outcome has been altered, but if, even in a few, life is comfortably prolonged, treatment with cortisone seems well worthwhile

Incidentally, with regard to the problem of infection, I may add that all of our patients, whether receiving cortisone or not, have been maintained on antibiotic prophylaxis throughout the active stages of their disease. While this has not wholly prevented infections, with resultant exacerbations, we feel that infections in general have been less frequent and less severe. Furthermore, on this regime, we almost never see the old familiar complications, such as peritonitis, any more.

CHAIRMAN BARNETT: Thank you very much, Dr. Guild.

I think, Dr. Danowski, you had some experience with adults that might be of interest if you would like to tell us about it.

DR. T. S. DANOWSKI: Dr. Greenman and other members of our group have treated fifteen adults with the nephrotic syndrome which we thought was of glomerulonephritic origin. The therapy was like that of the nephrotic children described by Dr. Greenman, except that we gave 200 milligrams of ACTH to almost all of these patients.

We gave nitrogen mustard to only 2 of these patients. The results in the 15 patients are nowhere near as good as they are in the case of the children, but they are nonetheless encouraging in view of the accepted hopeless prognosis for the nephrotic syndrome in adults. You will recall in the case of the children, 34 of the forty-one children that we treated are well; although we have had one or more flare-ups in about one-third of them. Therefore we have at any one time 83 per cent of the children in remission In the adult group 6 out of 15 are currently in remission.

Again we wish to point out that these people were put on a regimen of low sodium and high potassium intake. It may well be that ACTH given to such patients does more than ACTH given without sodium restriction and potassium supplementation. This is evident in the gross morphologic changes in the adrenal cortices described by Deane, W. H., et al, [1], and Badinez, O., [2] and others, as accompanying such regimens. We ourselves have measured neutral 17-ketosteroids in a number of patients. I would like to indicate the order of magnitude of response that is seen. For example, we have one patient who put out 132 milligrams of 17-ketosteroids in twenty-four hours, and we have another who excreted 94 milligrams in twenty-four hours. These amounts point to marked hyperfunction of the adrenal cortices in patients receiving ACTH on this regimen.

One final comment. Though we have not used nitrogen mustard in the bulk of these studies in adults you will recall that Dr. Greenman pointed out we can't recognize any additional beneficial effect attributable to mustard

DR. LANGE: We have had eight adults in our group of patients with the nephrotic syndrome Of the eight, seven are in remission following ACTH and one we have never been able to diurese He has had four courses of ACTH and four of nitrogen mustard.

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[1] Endocrinology 43: 133, 1948.
[2] Bol. Soc. biol. Santiago, 5: 40, 1948.

CHAIRMAN BARNETT: I wonder if anybody would care to discuss the question of the relative effectiveness of ACTH and Cortisone. Are there differences of opinion? Con, I think you have a definite opinion about it.

DR. RILEY: Simply that I feel they are interchangeable. I haven't been able to see any difference.

DR. HEYMANN: I showed our work in this respect to Dr Badger who is our statistician, and as you remember, Dr. Spector showed we had a difference in the length of remission between ACTH treated children and cortisone treated children. 4 6 months to 2.7 months, I believe.

He did not think our figures would lend themselves to statistical analysis, but going over them, he had the impression they suggested strongly that the lengths of remission after cortisone were shorter, with short-term treatment.

DR. SLATER: I would like to ask -- is the role of heparin in nephrosis known? Is it too dangerous a drug to use in children in carefully controlled doses? The rather favorable results in some animals in which nephrosis has been induced makes one think about the possibility of it as another form of therapy which hasn't been exploited.

CHAIRMAN BARNETT· I think both Dr Riley and Dr Lange had some experience with heparin

DR RILEY We tried it in two children with Dr Howard Eder following the lipids in the blood very carefully. We gave it, as I recall, for seven days in daily doses. Nothing happened either clinically or to the lipids in the serum.

DR LANGE In animals with the nephrotic syndrome, you can suppress the disease, or at least, ameliorate it almost completely if you give very large doses. For a rabbit you need about 80 to 100 milligrams a day, so the dose required is too high from the anti-coagulant viewpoint. With lower doses you don't get any effect

CHAIRMAN BARNETT· We considered it and spoke with Dr. Irving Wright who, as many of you know, is interested in anti-coagulant therapy He believed it would be too dangerous to warrant its use in nephrosis

DR. HEYMANN I very briefly want to stress one point which for lack of time was not emphasized in the data that Dr Spector presented.

Years ago, after chemotherapy was introduced, we reviewed a similar group of nephrotic children as the one that Dr. Spector discussed today.

The death rate is equal in both groups. One chemotherapy without hormones; the other chemotherapy and hormones.

CHAIRMAN BARNETT Having heard impressions of therapy, it is obvious that compilation of all our data in systematic fashion would facilitate needed statistical analyses The suggestion has been made that we might expedite the beginning compi-

TABLE 59 Composite adjusted life table (533 patients) comparing survival of untreated and steroid treated patients

	CATEGORY I - No Treatment					CATEGORY II - Treatment for edema only					CATEGORY II - Treatment by pre-planned schedule						
x Months after onset	lx	nx	wx	dx	qx %	lx	nx	wx	dx	qx %	lx	nx	wx	dx	qx %	qx* Exp. %	dx** Exp.
0-2	--	158	15	1	1 3	--	108	5	2	3.7	--	64	3	1	3 1	2.2	0 7
3-5	142	92	85	5	3 4	101	48	9	4	3 3	60	25	14	0	0	3.4	2 2
6-8	144	40	52	5	3 6	136	22	11	6	4.2	71	18	5	0	0	3.9	3 0
9-11	127	18	23	3	2 4	141	11	8	3	2.1	84	5	14	1	1.2	2 2	1.7
12-14	119	17	18	8	6 7	141	10	13	4	2 9	74	6	13	1	1.4	4.8	3 4
15-17	110	7	10	3	2.8	134	6	11	7	5 3	66	1	20	0	0	4.2	2.3
18-20	104	3	10	5	5 0	122	2	12	6	5.1	47	1	9	0	0	5.1	2 2
21-23	92	5	9	1	1.1	106	5	5	5	4.7	39	2	11	2	5 8	3.1	.9
24-26	87	5	8	2	2 3	101	5	6	4	4 0	28	0	7	1	4.1	3 2	8
27-29	82	1	5	3	3 7	96	1	4	6	6 2	20	0	3	0	0	5.2	1 0
30-32	75	1	5	1	1.4	87	4	6	3	3 5	17	0	6	0	0	2.5	.4
33-35	70	0	4	1	1 5	82	1	9	2	2 6	11	0	2	1		2 0	.2
36-41	65	5	7	2	3 1	72	2	8	2	3.1	8	0	2	0		3 1	.2
42-47	61	1	3	1	1.6	54	0	14	3	6 4	6	0	3	0		3.7	2
48-53	58	2	6	2	3.6	37	1	12	2	6.3	3	0	1	0			
54-59	52	1	3	1	2 0	24	0	6	0		2	1	1	0			
60-71	49	2	12	2	4.5	18	1	11	2		2	0	1	0			
72-83	37	3	17	0		6	2	4	1		1	0	1	0			
84-95	23	0	13	0		3	2	3	0		0	1	0	0			
96-107	10	1	4	1		2	0	0	0		1	0	1	0			
108-119	6	0	2	0		2	0	1	0		0	0	0	0			
120-131	4	0	0	0		1	0	0	0		0	0	0	0			
132-143	4	0	0	1		1	0	1	0		0	0	0	0			
144-155	3	0	0	0		0	0	0	0		0	0	0	0			
> 155	3	0	2	1		0	0	0	0		0	0	0	0			

Key
- x - 3 months intervals after onset of edema
- lx - individuals living at beginning of interval
- nx - new individuals entering during interval
- wx - individuals withdrawing during interval (lost to follow-up, currently in interval, transferred to another category).
- dx - individuals dying during interval
- qx - death rate per interval - $\frac{dx}{lx + 1/2nx - 1/2wx}$

*Average (expected) qx as calculated from a pooling of data of I and II.

**Expected dx calculated on basis of expected qx.

NOTE. Up to 30 months after onset indicated by single line in expected qx and expected dx column. Total expected deaths = 18.2. At same time after onset, actual observed deaths = 6 χ^2 of difference 7 6

lation of data regarding effects of steroid therapy if the form Dr. Riley has prepared could be completed for each patient under the care of each doctor in this room. If this were done promptly, the results could actually be included in the transcription of this conference. Just looking at the number of patients that were presented in the last hour and a half, there are well over 500 patients being taken care of by people in this room right now.

I think we really need this information urgently, and from what Dr. Metcoff said, I think Dr. Riley indicated that he would be willing to analyze them. Is that true?

DR. RILEY: Yes. (The results of this compilation follow).

PRELIMINARY REPORT OF STATISTICAL EVALUATION OF EFFECT OF ADRENOCORTICAL-ACTIVE HORMONES ON CHILDHOOD NEPHROSIS

Prepared by Dr Riley and Associates
Based on Responses to the Questionnaire

To the date of writing (January 14, 1955), 533 such questionnaires suitable for evaluation purposes have been analyzed on patients whose illness began below the thirteenth year of life. Since few reports have been received on adults, it has been decided to set those aside and treat them as a separate category Other questionnaires are still being returned, so that a complete report will be given at a later date.

In the current analysis the following considerations were used in entering cases and withdrawing them from the accompanying life tables. The time intervals (x) during the first 3 years after the onset of edema are of 3 months each. Cases with no adrenocortical-active therapy were entered as new cases (N_x) in the intervals when they were first seen by the reporting physician. They were withdrawn (W_x) at the proper interval, either at the time when observation of them ceased or when one of the forms of hormone treatment under discussion was started Cases in the "treatment for edema only" and "treatment by schedule" categories were entered (N_x) at the interval when such treatment was first started Cases in which such treatment was administered by some other physician prior to their observation by the reporting doctor were not included Also, cases in which treatment was given first for edema only and later by schedule, unless the interval between these two was one month or less, were excluded. If enough such cases later become available, a separate category will be made for this group.

We have carefully reviewed our technics with Dr John Fertig, Professor of Biostatistics at the Columbia University School of Public Health, and are most grateful for his advice and criticism Certain glaring weaknesses in such an analysis should be pointed out. First, the cases being studied are not contemporaneous, in fact, Category I (no treatment) in general tends to antedate Category II (treatment for edema only) which in turn represents an earlier group than Category III (treatment by schedule) Thus, the "controls" are not comparable in period of time to the group being specifically tested. One easily discernible difference which such difference in time might make, control of infection, has been minimized by accepting only cases observed since

the beginning of 1946 when effective anti-bacterial therapy was an accomplished fact.

A second objection is that cases in which treatment was first given early are frequently compared to cases in which treatment was not started till late in the disease. That this can make some difference, usually by unfavorably altering the mortality rate, has been shown by small-scale separate analysis of the early and late treated cases. It is hoped that as more figures become available such separate analyses can be made on a large scale

With those qualifying remarks, the accompanying life table (Table 59) is submitted. The technic for calculation of the probability of dying in a given interval (Q_x) was reported at the conference and is accepted statistical practice.

A preliminary glance at survival times in Categories I and II, calculated from the Qx's, showed that there was no significant difference between them in the first 2 1/2 - 3 years after onset. Therefore, these two were pooled to form a "universe" with which Category III could be compared. Here it is necessary again to point out a qualification - this "universe" is not yet large enough to have its rates for probability of dying in any single interval stablized.

Next, this probability of dying, as calculated from the "universe", was applied in each interval to the cases in which treatment had been given on pre-planned schedule. At the end of 30 months after onset had these rates obtained, there would have been 18.2 deaths. The observed deaths were 6. Chi square, calculated for this difference, is 7 6, indicating a P of less than 0.01. If the P is calculated on the basis only of patients starting treatment in the first 6 months of the disease, it becomes less than 0.05. This change in P value appears to be the result of reduction in total numbers, but the collection of further data should help to determine this. The reason for making the calculation at 30 months after onset is that treatment by schedule has not been given much more than this length of time.

With the qualifications discussed, no final conclusions are justified. One can only report that the evidence for the fact that scheduled treatment prolongs life in childhood nephrosis is suggestive.

CHAIRMAN BARNETT: I think the major question of whether or not these hormones are favorably affecting the final outcome of this disease cannot be answered with assurance until further evaluations are made along lines of what Dr. Riley has said. My own impression is that the chance that they are favorably affecting the disease are good enough that I certainly will continue to use the hormones.

DR. JANEWAY: There are two or three things I would like to say, rounding this out. In the first place, Dr. Rapoport left me a note when he left and said he was afraid he hadn't made himself 100 per cent clear, and he wanted to be sure people understood his point. I think he did, but basically, it is that really there are, two groups, those who respond very nicely in terms of becoming protein free in class one, and those that don't get protein free in class two, even though you keep up cortisone twice as long as you need to. He feels that all of these adjunctive methods of treatment like long-term maintenance therapy, should probably be restricted to this second group, in which the prognosis is definitely much worse, and in which ultimate outcome of the disease is much more in doubt.

Another thing I would like to mention because his group isn't represented here, is that when I was out in Minnesota a couple of weeks ago, Dr. Watson told me among adults they had found therapeutic malaria was the most effective means for treating the nephrotic syndrome of anything they had ever tried. It worked better than ACTH. So here is a stressful reaction with high fever in an infectious disease which you can turn off at will with chemotherapy. He was very much excited about it, so this is another thing to keep up your sleeve for the future.

There are certain things that are going to come out of this, with Dr. Riley's analysis, and I am delighted we are going to do this cooperative job. It seems to me all through this meeting there was a pretty clear indication that perhaps the people who were treating the remission of the disease per se have been doing better than those of us who restricted ourselves to what you might call symptomatic treatment for a manifestation of the disease

There certainly is a spectrum of case response in nephrosis. At one end of the spectrum, the patient will get well no matter what you do. At the other end of the spectrum, the patient probably wouldn't recover no matter what you do. I am reminded of our experience with inoculated measles which was much, much smaller. Again, we never saw a good measles diuresis in a patient who obviously fell into this group at the bad end of the spectrum. We didn't have half the data then on patients which we have today, but I think the similarity of response in general, no matter what kind of treatment you are using, is really pretty striking.

It is an awfully good thing to remind ourselves that, as Dr. Guild suggested, we may be making a lot of these patients more comfortable and possibly rendering them safer from infection by steroid treatment because they are non-edematous At the same time, we must remember that patients recovered from this disease in about 35 to 40 per cent of the cases, if they didn't die of acute infection, long before the steroids were known or introduced. Basically, there is not much evidence yet we have changed that figure.

I think we have got to be awfully skeptical and remember that Dr. Spector exhibited some rather serious complications of steroid therapy. All of us who have had any large experience with steroid therapy have encountered this. No matter how carefully we watch, people do get into trouble with these drugs which are awfully potent Granted they got into trouble with the disease before we had the drugs, but it is certainly not a simple form of treatment.

DR LUETSCHER· Could I comment on two points? First, we have been fortunate at Stanford because of the encouragement of Drs. Faber, Anderson, and Piel to work in pediatrics as well as in the adult medical wards. This has allowed a comparison [illegible]

short-term therapy in the youngsters and in the adults Just short of one-half of each group recovered completely

The second comment concerns the question of treating on indication versus treating on schedule. Although scheduled treatment certainly offers many points of superiority, still until we have a completely safe and effective form of therapy, we ought to keep in mind the hazards of high dose, continuous steroid therapy. If we embark on a long-range, high dose schedule in every patient, we are going to induce some unnecessary trouble in a certain number of patients, since half of these patients would have gotten well with much less treatment.

For this reason, at the moment, we are taking an intermediate position, and are using a few short courses of therapy to see where we stand. We feel secure in this approach in the patient who is seen very early, but not in the patient who is beginning to deteriorate If the patient fails to respond promptly, if there is prompt relapse, or if there are signs of glomerular involvement of a greater severity, we would feel that patient ought to be given enough ACTH or cortisone to induce maximal improvement and then put on a prolonged course of scheduled treatment.

VII. REPORT ON WORK IN PROGRESS

CHAIRMAN BARNETT: I think I would like now to turn to brief descriptions of some of the work that is currently going on, and perhaps Dr. Hodges and Dr. Metcoff and Dr Kramer would share the remaining twenty-five minutes or so. If there is any time left, other people might tell us of work in progress. Dr. Hodges, would you care to start?

DR. RICHARD HODGES We were interested in the response of nephrotic children to an antigenic stimulus We took 8 nephrotic children in the edematous stage. Serum was obtained and type specific polysaccharides of the pneumococcus types I & II were injected The children were bled again 2 weeks later. We had 11 non-nephrotic children of comparable age who were treated in the same manner. The paired sera were then tested by the mouse protection test and results are expressed in terms of that test.

The non-nephprotic children tested with type I pneumococcus had some natural immunity, but the immunization, the injection, greatly increased that immunity

The response in the edematous, nephrotic patients was nowhere near as great

When the control group was tested against type II pneumococcus, the antigenic response as far as the mouse protection test went was very good. Following this antigenic stimulus, the level of antibody in nephrotic children was not nearly as high as it was in the control group Only one of the nephrotic children really resembled the control group in her response. Interestingly enough, she was the only one that had a diuresis between the times of two blood drawings.

DR JANEWAY You haven't got subsequent sera on the other nephrotic patients after diuresis? I bet they go way up.

DR HODGES: I will bet they do, too.

CHAIRMAN BARNETT. Dr Metcoff.

DR METCOFF. Dr Sylvester Frenk, Miss Antonowicz, Dr John Craig and I have been exploring the nephrotic syndrome in rats induced by a new technique, the daily subcutaneous injection of 6 dimethyl amino purine, 0-3 amino α-ribose, an ammonucleoside representing a hydrolysis product of the antibiotic "puromycin" - given

for 12 days. Rats develop marked edema, ascites, hyperlipemia, hypoproteinemia, proteinuria, but no frank hematuria. They do have azotemia. I might preface my remarks by saying that we do not yet know the natural history in detail of this kind of rat disease. We have not had time to explore it. We hope that the material will become available, and that enough investigators will be able to study the disease and determine its features.

Figure 104 is a picture of two pair-fed animals killed at the end of 14 days of pair feeding and after 12 days of drug injection. The control animal is on the left. They have been depilated prior to sacrifice to facilitate tissue analyses.

In the nephrotic animal the renal lesions consist of thickening of the basement membrane of the glomeruli, and the tubules when stained for fat show a marked increase in the neutral fat droplets at the basal ends of the tubular cells. There is also an alteration in the phospholipid pattern in the tubule cells.

In a recent experiment we pair-fed 18 young rats, during development of the nephrotic syndrome, with 18 controls. Twelve in each group were loaded by gavage with sodium chloride in a dose of 10 millimols per kilogram body weight - a standard loading dose - the last 4 days of the experiment. Muscle, entire skin, and total carcass were analyzed in all 36 rats. The results show that there is a slight decrease in the intracellular potassium concentration of muscle when corrected for collagen and expressed as non-collagenous, dry fat-free solids. This decreased intracellular potassium concentration is due to intracellular edema, since the potassium content of muscle is not decreased.

Skin, which normally accounts for 15-18% of total body weight, appears to be the principal tissue reservoir of edema fluid.

The total residual carcass contains a smaller quantity of tissue edema fluid. Approximately 1/2 of the edema accumulates as free ascitic fluid.

We are engaged currently in initiating some simultaneous electrolyte balance, tissue composition and isotope studies to determine the specific activities of the electrolytes particularly in the skin since there has been some suggestion made that there is not complete excqangeability of sodium or chloride in this organ. We are hoping to learn also whether isotope distributions compare favorably with indirect balance and direct tissue analytic techniques. Ultimately we plan to explore other features of fat, protein and electrolyte metabolism in these animals in order to project meaningful studies to the only valid test subject - the human patient with nephrotic syndrome.

CHAIRMAN BARNETT: Thank you, Dr. Metcoff. This new rat disease opens further investigative possibilities. We will now proceed to Dr. Kramer.

DR. KRAMER: The object of our study was to determine the mechanism of some of the phenomenon commonly seen in nephrosis, namely, the hyperproteinemia, hyperlipemia and hypercholesteremia.



Fig. 104

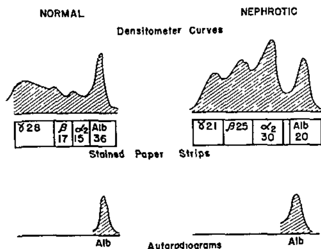


Fig 105

Fig 104. Nephrotic rat on right. Pair fed control on left. Numbers are body weights. Animals depilated.

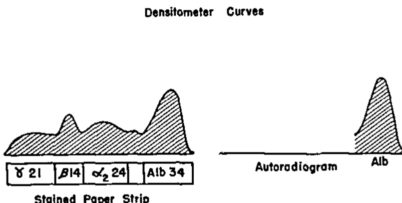


Fig. 106

Fig. 105
Migration of administered I^{131} human serum albumin with <u>in vivo</u> circulating albumin shown by paper electrophoresis and radioautography

Fig 106. Distribution of radioactivity in proteins of nephrotic urine following administration of I^{131} human serum albumin

For this purpose we studied the rate of disappearance of iodine 131 albumin from plasma when injected intravenously. We also studied the rate of synthesis of proteins using labeled glycine, and the rate of formation of cholesterol by using tritium labeled sodium acetate, and the rate of disappearance of cholesterol using cholesterol for C-14.

What I want to show today are the results using the iodine 131 labeled albumin.

Figure 105 is a paper electrophoresis pattern of the normal and nephrotic child's serum and shows the usual picture but in addition it shows that when the iodine 131 albumin is given the activity moves with the albumin and corresponds to the albumin of the subject, indicating that the albumin is undenatured in this process.

Similarly, Figure 106 shows the electrophoresis pattern of the urine of the nephrotic patient and again the activity is in the albumin fraction.

When we studied the accumulative excretion of the isotopic albumin, (Fig. 107), we found the rate of excretion was very much greater in the nephrotic than in the normal subject.

When we studied the rate of disappearance of the radioactivity in the plasma, (Fig 108) we found that this was much faster in the nephrotic than in the normal. The half-life in the normal was about 12 days, whereas, in the nephrotic it was between 2 and 2 1/2 days

Now, we haven't as yet studied the rate of synthesis, but if the concentration of albumin in the serum remains unchanged, we have tentatively assumed that this indicates a rate of synthesis of albumin about 5 or 6 times as fast in the nephrotic as in the normal subject

We had one case of congenital hyperlipemia without albuminuria, and this child showed a rate of disappearance which was twice as fast as the normal subject

We then studied the distribution of proteins and lipids in nephrotic serum and urine by electrophoresis (Fig. 109). We were unable to find any free lipid in the urine, but found the lipid moving along with the alpha-2 and the beta-lipoprotein.

Tentatively, we had worked on the hypothesis that the rate of synthesis of the various prot [illegible]
evidence for [illegible]
since the k [illegible]
beta-lipoproteins, there is accumulation of these materials in the blood which may account for the hyperlipemia and perhaps the hypercholesterolemia. Active isotopic cholesterol given intravenously could not be detected in the urine, although larger amounts were present in the plasma.

CHAIRMAN BARNETT. Thank you, Dr. Kramer. I am sure that there are a lot of people here who would like to discuss this, but because of the time limitations I think perhaps we should now ask Dr. Janeway to close the meeting.

DR. JANEWAY: You always have to have a start and finish to these things. I will be as brief as I can.

On behalf of all of us who have stuck with this conference now for the six years we have had them, I would like to say thank you to everybody here who has participated. I think you would all agree with me that this has been an extremely interesting, stimulating and exciting meeting. It is due to the fact that people have come here and talked very frankly about their work, and because, in a small room, even though our remarks are being taken down, people have been willing to go far out on a limb speculatively at times, which really makes this sort of thing fun.

I think it is worth-while also expressing our thanks to the National Nephrosis Foundation, which is making it possible to take down a stenotypic record of this meeting and to get it distributed.

We plan, through Dr. Metcoff, to get the proceedings published promptly.* We plan likewise to perhaps publish a very brief summary which will be written up by Dr. Heymann to be published as a special report in Pediatrics, and we hope that perhaps in another year or two all the expenses of this meeting can be met by the Foundation. As it is, they are meeting a good part of them.

I would also like to express gratitude to all of you who cooperated in providing the figures Dr. Riley gave us and showed that this is a cooperative venture. One of the objectives of this meeting is to speed our own research up by meeting here and sharing what we are doing In regard to treatment particularly in a disease like this, where we do have effective agents but no agreement on how to use them, this kind of cooperation is the kind of thing that can go on in this country and probably not in many other parts of the world, and it is one of the things we are proud of. I think it will be an extremely useful contribution to medicine and pediatrics that we can share our experiences and make them available to the people who have to practice and take care of patients.

Finally, I would like to say thank you to Dr. Heymann and the Cleveland group. I think he has indicated by the content of the program that this is a very exciting place to be medically. There is a lot going on that is interesting and stimulating. He has planned a wonderful program and certainly we have had the best of hospitality. I want to thank him for all of us for the great amount of work he did to make this meeting a success

*Copies available from The National Nephrosis Foundation, at $3.50 per copy.

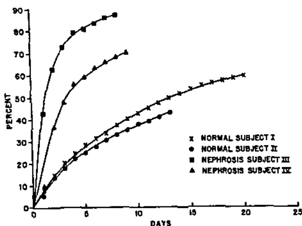


Fig. 107

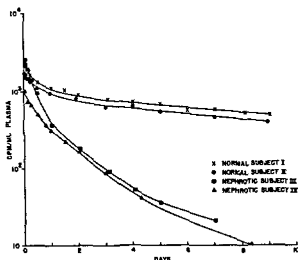


Fig. 108

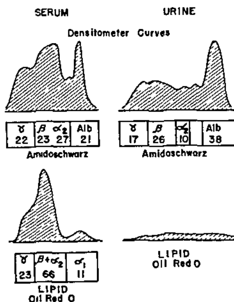


Fig. 109

Fig 107 Cumulative urinary excretion of radioactivity following the administration of I^{131} human serum albumin to normal and nephrotic subjects

Fig 108. Disappearance of I^{131} human serum albumin radioactivity from plasma of normal and nephrotic subjects

Fig 109 Distribution of proteins and lipids in nephrotic serum and urine shown by paper electrophoresis and selective staining